Bioorganic & Medicinal Chemistry 19 (2011) 3669–3677

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and biological activity of splitomicin analogs targeted at human NAD$^+$-dependent histone deacetylases (sirtuins)

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ARTICLE INFO

Article history:
Received 28 September 2010
Revised 7 January 2011
Accepted 13 January 2011
Available online 22 January 2011

Keywords:
Benzimidazole
Histone deacetylases
Ligand efficiency
Sirtuins
Splitomicin

ABSTRACT

Small molecules interfering with posttranslational modification of histones are of interest as tools to study epigenetic regulation of gene transcription. Specifically, drugs that interfere with histone deacetylation could be useful to induce differentiation, growth arrest as well as apoptotic cell death in tumor cells. One class of histone deacetylases is known as sirtuins some of which (*Saccharomyces cerevisiae* Sir2) are for example inhibited by the lactone splitomicin leading to telomeric silencing in yeast. However, splitomicin is only a micromolar inhibitor of yeast Sir2 and does not inhibit human subtypes and the lactone is prone to hydrolytic ring opening. In preliminary SAR-studies, splitomicin analogs lacking this hydrolytically labile ring were described as inactive while the naphthalene moiety could successfully be replaced by smaller aromatic rings in a fragment-like dihydrocoumarin. Here we report the synthesis and biological activity of a series of hydrolytically stable analogs with activity against human SIRT1 and 2. These comparatively small compounds characterized by high ligand efficiency are used as a starting point toward the development of specific inhibitors of histone deacetylases from the class of sirtuins.

1. Introduction

The lactone splitomicin (**1**) was purified from a mixture of compounds (a test sample named NSC-112546) containing cambinol as major constituent and reported to be a micromolar inhibitor of yeast Sir2p[1] (IC_{50} = 60 μM), an NAD$^+$-dependent deacetylase required for chromatin-dependent silencing in yeast. This cell-permeable molecule **1** is comparatively small in terms of the number of rings and a molar mass (i.e., below 200) and thus represents an attractive lead for novel compounds intended as modulators of histone deacetylase (HDAC) activity. Three of the four classes of human HDACs known to date consist of zinc-dependent amidohydrolases that cleave off acetyl groups from acetyl-lysine residues in histones[2] and various nonhistone proteins. The seven members of the non-zinc dependent class III HDACs named sirtuins for their homology to yeast silent information regulator Sir2p,[1] utilize nicotinamide adenine dinucleotide (NAD$^+$) for their catalytic activity. Recently, small molecule inhibitors of sirtuins have become valuable tools[3] for the investigation of sirtuin function[4] and have provided insight into their role in many physiological and pathological processes. Specifically, sirtuin activity has been associated with the pathogenesis of cancer,[5] HIV,[6] and metabolic,[7] and neurological[8] diseases. Within this context, sirtuin inhibition might offer an important novel starting point for therapeutic intervention. A first example of a potent and selective SIRT1 inhibitor (IC_{50} = 98 nM), EX-527 (**19**) has entered phase I clinical trials in 2010[5] as a drug for the treatment of Huntington's disease. The sirtuin family is thought to contain key players in the control of longevity in response to caloric restriction in organisms such as yeast,[9] worms, flies, and possibly mammals, thus both inhibitors[10] as well as activators are of potential interest as future drugs. The question whether sirtuins should be inhibited or activated is far from being settled, and novel sirtuin inhibitors with varying selectivity are of research interest in order to establish a set of well-defined pharmacological tools for further studies in this direction. Optimally, research could lead to the discovery of arrays of highly selective sirtuin inhibitors that could be classified as molecular probes.

While **1** is commercially available as a tool for experimental studies, it does not inhibit human sirtuin subtypes such as SIRT1 and is prone to hydrolysis[11] in aqueous media. The resulting ring open form **2** is characterized by a profound loss of activity. The straightforward replacement of the labile lactone functionality in **1** by a lactam function in analog **3** as depicted in Figure 1 led to a significant loss of activity[11] in the available assays. In preliminary structure–activity relationship (SAR) studies on a limited number of analogs, these results were interpreted in such a way that the

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doi:10.1016/j.bmc.2011.01.026

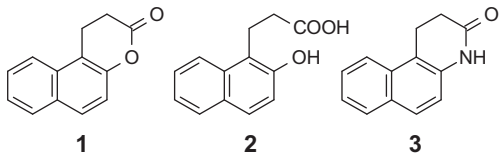


Figure 1. Splitomicin (**1**), inactive hydrolyzed form **2** and inactive stabilized analog **3**.

lactone ring was an essential part of the pharmacophoric structure. In order to verify this notion and with the aim to identify a novel lead structure with good stability and activity against human sirtuin subtypes, it appeared rewarding to synthesize and test additional analogs of **1** with other stable heterocyclic rings as replacements for the dihydropyran or dihydropyridine ring.

2. Chemistry

With the intention to identify stable analogs of lead compound **1**, the 2-oxo-substituted dihydropyran ring was formally replaced by a 2-oxo substituted dihydroimidazole ring. The selection of dihydroimidazole was based on the fact that this structural motif is present in many biologically active compounds and can be called a privileged structure in medicinal chemistry. In this way, the lactone ring was replaced by hydrolytically stable cyclic urea functionality resulting in compound **4**[12] that had already been prepared by Diels in 1922. Therefore, naphthalene-1,2-diamine was treated with triphosgene as described. In addition, as described before, compound **4** could easily be transformed regioselectively into the nitro derivative **5**[13] by nitration with nitric acid in glacial acetic acid (Scheme 1) in good yield.

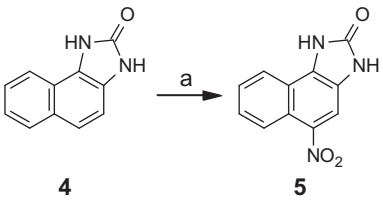
The regioselectivity of the above reaction enabled the straightforward purification of a single product. The structure of the known single major product **5** could be verified by HSQC- and HMBC-NMR experiments. Cyclization of naphthalene-1,2-diamine by means of CS_2 yielded related thiourea **6**[14] in a mixture of tautomers (**6a**–**c**, Fig. 2).

It was evident from preliminary SAR investigations that the annelated naphthalene moiety in **1** could be replaced by a benzene ring[11] leading to active dihydrocoumarin **7**, thus biological evaluation of smaller splitomicin analogs **8** and **9a**–**c** (Fig. 3) were envisioned as well.

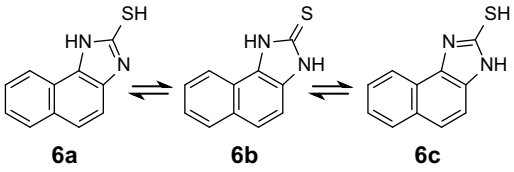
The known compounds **8**[15] and **9a**–**c**[16,17] were prepared according to procedures described in the literature.

Another known aspect of SAR within the splitomicin class of compounds is the fact that substituents such as aryl rings on both of the methylene groups of the lactone ring can contribute to improved activity[6,18] in selected assays. Therefore an additional series of N-substituted fragment-like compounds **10a**–**h** (Fig. 4) were prepared. The introduction of residues R^2 via nucleophilic aromatic substitution was aided by electron withdrawing substituents R^1.

The seven novel compounds **10a**–**g** were prepared from Sanger's reagent (**11**, X = F) with five aliphatic amines[19–21] yielding anilines **12a**–**e**. Subsequent regioselective reduction of the nitro



Scheme 1. Regioselective nitration of compound **4** to nitro-derivative **5**. (a) HNO_3/AcOH, 50 °C, 76% yield.



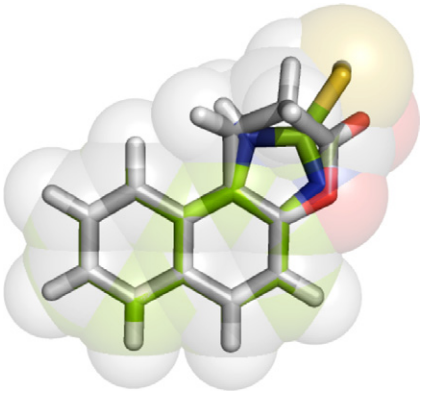


Figure 2. Known 1,3-dihydrobenzo[*e*]benzimidazole-2-thione (green) as thioenol tautomer **6a** as overlay on splitomicin (**1**, gray).

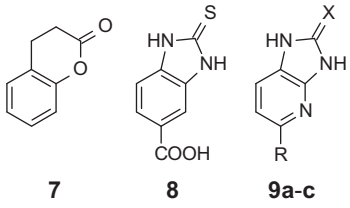
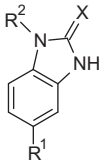


Figure 3. Active dihydrocoumarin **7** and general structure of analogs of type **8** and **9** (**9a** X = O, R = Cl, **9b** X = S, R = Cl, **9c** X = S, R = OCH_3).

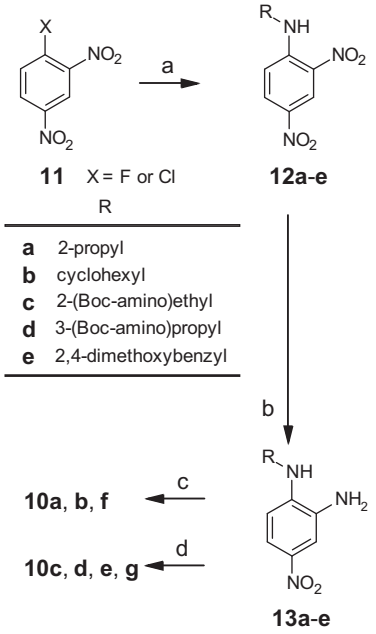


10a-h

	X	R^1	R^2
a	O	NO_2	2-propyl
b	O	NO_2	cyclohexyl
c	S	NO_2	2-aminoethyl HCl
d	S	NO_2	3-(Boc-amino)propyl
e	S	NO_2	3-aminopropyl HCl
f	O	NO_2	2,4-dimethoxybenzyl
g	S	NO_2	2,4-dimethoxybenzyl
h	O	CF_3	1,3-benzodioxol-5-yl

Figure 4. Structure of N-substituted analogs **10a**–**h**.

group adjacent to the newly introduced substituent of these intermediates gave the *o*-dianilines **13a**–**e** as outlined in Scheme 2. The necessary regioselective reduction can be achieved by using baker's yeast[22] and many other reducing agents. In our hands, the established method with $NaHCO_3/Na_2S$[23] worked best. The boiling methanolic solutions of **12a**–**e** were treated with an aqueous equimolar solution of $NaHCO_3$ and Na_2S and refluxed for several hours. The resulting 4-nitro substituted 1,2-dianilines **13a**, **b**, and **e** could be cyclized by means of triphosgene to form the cyclic ureas **10a**, **b**,

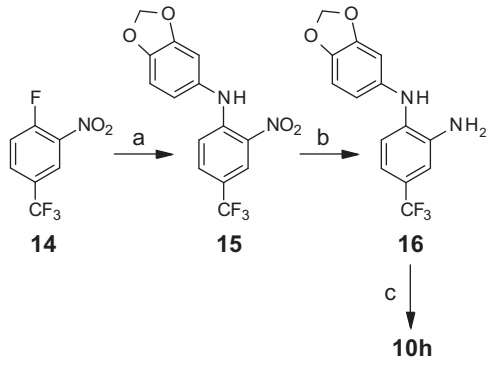


Scheme 2. Synthesis of N-substituted analogs **10a**–**g**. Reagents and conditions: (a) R-NH_2, EtOH or acetone/H_2O/$NaHCO_3$ 125 °C or reflux; (b) MeOH, reflux, $NaHCO_3$, $Na_2S{\cdot}9H_2O$, 5 h; (c) CH_2Cl_2, NaOH, H_2O, triphosgene, 12 h; (d) CS_2, Et_3N, EtOH, reflux, 4–5 h.

and **f**. The thioureas **10d** and **g** were obtained from the corresponding 4-nitro substituted 1,2-dianilines **13c**–**e** after cyclization with CS_2. Compounds **10c** and **e** were prepared via their Boc-protected precursors. The Boc-protected precursor **10d** of compound **10e** was included in the biological evaluation.

Compound **10h** bearing a trifluoromethyl group in position 5 was obtained from 1-fluoro-2-nitro-4-(trifluoromethyl)benzene (**14**). In this case, an aromatic amine, 1,3-benzodioxol-5-ylamine was selected for the introduction of an aromatic substituent in the iminodibenzyl derivative **15**. The absence of a second nitro group in position 4 of this intermediate allowed for the reduction of the nitro group in **15** to form commercially available 1,2-dianiline **16** via straightforward catalytic hydrogenation (Scheme 3).

The six different amines were selected to introduce flexible alkyl chains with or without polar substituents as well as space filling aliphatic and aromatic rings into the molecular scaffold in order to generate additional interaction motifs. The resulting substitution pattern of compounds **10a**–**h** meets the hypothesis that substituents on one of the two N-atoms might occupy the same space as the beneficial substituents on the methylene groups of related compounds.[6,18] The annelated naphthalene moiety in **1** could be omitted to yield active derivative **7** as mentioned before and thus we anticipated that an additional gain of affinity might be best obtained by substituents in a different hitherto unexplored position.



Scheme 3. Synthesis of N-substituted derivative **10h**. Reagents and conditions: (a) 3,4-(methylenedioxy)aniline, neat, 80 °C; (b) Pd/C 10%, H_2, EtOH; (c) CH_2Cl_2, NaOH, H_2O, triphosgene, 12 h.

3. Results

The seven known splitomicin analogs **4**–**6** and **8**–**9c** were evaluated for sirtuin inhibition together with the ten novel N-substituted derivatives **10a**–**h**. The biological activity was determined by using a homogeneous microplate reader-based nonisotopic histone deacetylase activity assay[24] as described before. The results of the sirtuin assay with human recombinant proteins produced in *Escherichia coli* are shown in Table 1. All compounds were tested at concentrations of 25 and 50 μM. For those that showed a significant inhibition, IC_{50} values were determined.

Neugebauer et al. reported the synthesis and biological evaluation of a series of β-arylsplitomicins[18] like **17** (Fig. 5). An investigation of the structure–activity relationships led to the discovery of SIRT2 inhibitors with single digit micromolar potency, and a selectivity for SIRT2 over SIRT1. In order to establish the binding mode

Table 1
Comparison of the biological activity of splitomicin analogs (IC_{50} ± SE [μM] or inhibition @ 50 μM).

Entry	SIRT1	SIRT2	SIRT3
Ro 31-8220	3.5 ± 0.4	0.8 ± 0.2	3.7 ± 0.2
4	10.6%	46.7%	24.6%
5	n.i.	12.0 ± 2.1	n.i.
6	9.6 ± 1.0	10.0 ± 0.7	n.i.
8	29.3 ± 2.9	38.3 ± 3.7	n.i.
9a	n.i.	n.i.	n.i.
9b	>100	26.9 ± 6.4	n.i.
9c	21.4 ± 1.0	16.3 ± 2.0	n.i.
10a	n.i.	n.i.	n.i.
10b	n.i.	34.4 ± 11.0	n.i.
10c	7.7 ± 1.2	5.0 ± 1.1	n.i.
10d	10.0%	n.i.	n.i
10e	n.i.	n.i.	n.i.
10f	n.i.	n.i.	n.i.
10g	14.4%	43.1%	n.i.
10h	n.i.	16.5%	n.i.
12e	n.i.	14.8%	n.i.
13e	14.1%	n.i.	12.0%
15	18.8%	41.4%	n.i.

Reference inhibitor Ro 31-8220 was tested in the same system.[3,25]
n.i.: no inhibiton [<10% @ 50 μM].

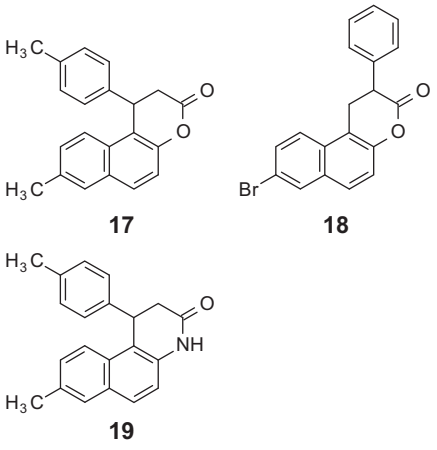


Figure 5. Labile lactone-based racemic splitomicin (**1**) analogs **17** and **18** (HR73) and stable lactam **19** reported by Neugebauer et al.[18] and Pagans et al.[6]

of these compounds and to rationalize the SAR of this series of SIRT2 inhibitors, docking simulations were performed.[4] The docked β-arylsplitomicin **17** revealed a binding mode that includes a hydrogen bond to a water network around Gln167, similar to the binding mode[26,27] of the major constituent of NSC-112546, cambinol. The β-phenyl substituent of the (*R*)-enantiomer of **17** fits into a hydrophobic channel and is sandwiched between Phe119 and His187. The (*R*)-enantiomer of **17** was shown to be a potent SIRT2 inhibitor with an IC_{50} of 1.0 μM but the (*S*)-enantiomer displayed only weak SIRT2 inhibition at high concentrations. While the stereochemistry at the substituted β-carbon atom is of great importance, the shift of the substituent from the β- to the α-carbon as well led to the identification of similarly active sirtuin inhibitor named HR73[6] (**18**). However, the notorious limitation of the instability of the lactone ring could be overcome neither by α- nor by β-arylsplitomicins, but within the series of analogous compounds, a stable lactam (**19**) could be shown to be equally active.[18] Docking studies of these compounds have given insight into the unknown binding mode of, for example, compound **6** in its three tautomeric forms in the nicotinamide binding pocket of human SIRT2, as suggested in Figure 6.

While tautomers **b** and **c** of compound **6** bind in virtually identical position (the magenta colored skeleton of **6c** is almost completely overlapped by light blue molecule **6b**, only the thiol hydrogen atom protrudes from underneath the thion sulfur of **6b**), a preferred docking solution for **6a** suggests that this enethiol tautomer with a hydrogen atom on N1 is shifted significantly towards Leu134 and Phe119.

The replacement of the six-membered tetrahydropyran ring of splitomicins **1**, **17**, and **18** by the five-membered imidazole leads to a conformationally closely related smaller ring system with cyclic urea structure. While the resulting ureas are less prone to hydrolysis, the N-substituents might interact with the molecular target similar to splitomicins **17** and **18** so that substituents on the N1 atom of the imidazole ring possibly can fit into the same gap between Phe119 and His187. The cyclic ureas or thioureas and their tautomers also could interact by a hydrogen bond network with conserved water molecules in the region of Gln167 and form hydrogen bonds with His187. The rationale for the synthesis of compounds **10a**–**h** was the fact, that modifications (a–d) could be combined in molecules of this type. Various substituents could easily be introduced into position 1 of the ring system by high yielding nucleophilic aromatic substitution. In contrast to molecular framework of α- or β-substituted derivatives **17** and **18** of **1**, no stereocenters are formed by this operation which is an advantage with respect to the ease of preparation. Because no

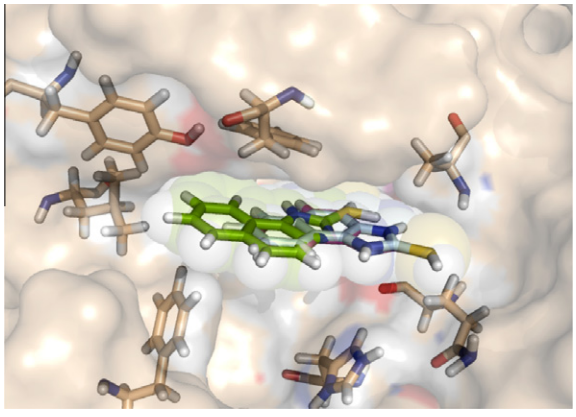


Figure 6. Predicted binding mode of the three tautomers of **6a** (green), **6b** (light blue), and **6c** (magenta) to human SIRT2 (1 J8F.pdb) in analogy to published docking simulations for **17**.

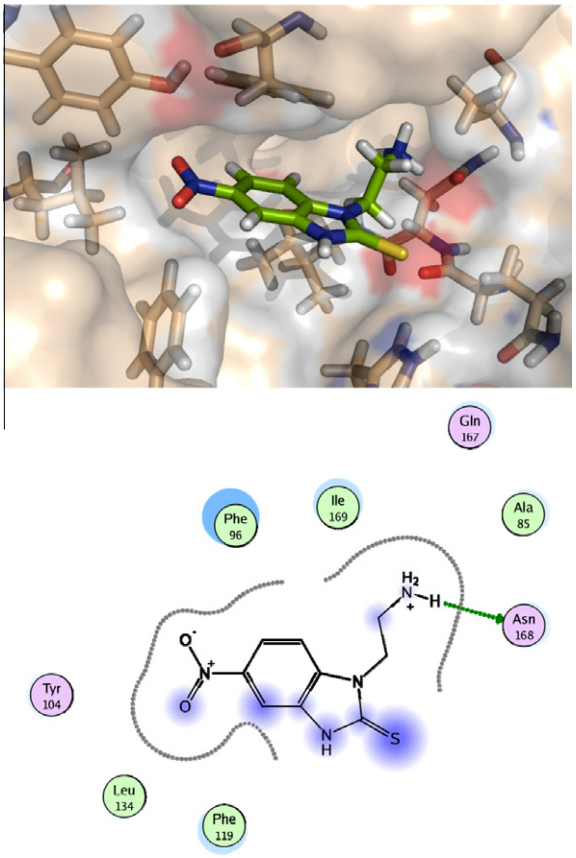


Figure 7. Predicted binding mode and molecular interactions for novel lead structure **10c** in the thione form.

X-ray structure of sirtuins with the compounds **5**, **6** or **10a**–**h** exists to date, the proposed binding mode and molecular interactions depicted in figure 7 remain speculative. According to docking simulations the binding mode of **1**, **6**, and **10c** could be similar in terms of the orientation of the carbocyclic part of the scaffold in the channel between Phe96 and Phe119. The water network between Gln167, Asn168 and the lactone or thiourea moiety of **1** or **6**, respectively, could be strengthened by the polar side chain of **10c** as suggested below. However, multiple binding modes are possible and **10c** could as well be flipped with the core aromatic ring system and the N1 substituents changing positions as recently reported for a biphenyl substituted thiobarbiturate.[28]

Due to the fact that the N1 atom is blocked by a substituent, only two tautomeric forms of **10c** are possible.

4. Discussion

Starting from the cell-permeable lead structure **1** that is inactive against human sirtuins and prone to hydrolytic lactone ring opening leading to a short half-life particularly at physiological pH, stable analogs with inhibitory activity in the selected enzyme were discovered in the present study. While the first analog **4** of similar size as **1** was practically inactive towards SIRT1 and only showed moderate inhibition of SIRT2, the introduction of a nitro group in position 5 of the 1,3-dihydrobenzo[*e*]benzimidazol-2-one ring of **4** lead to a more potent derivative **5** with an IC_{50} value of 12.0 μM for SIRT2. An even more pronounced effect could be observed after formally exchanging the oxygen atom in **4** by a sulfur atom in compound **6**. Interestingly, the SIRT2 selectivity in **5** is completely lost in **6**. The IC_{50} value of this spitomicin analog was 10.0 μM for SIRT2 and 9.6 μM for SIRT1. The question whether this

increase in SIRT1 inhibition may be attributed to the fact that the thiourea **6** is able to form enethiol tautomers thus altering hydrogen bond network architecture or by size or acidity remains to be investigated. Both compounds **5** and **6** already show considerable improvement over compound **1**. As well (a) the 5-nitro-substitution as (b) the exchange of the urea moiety by a thiourea function on the one hand appear to be beneficial modifications of **4** but lead to an increase of molecular mass and potentially a decrease of ligand efficiency and cell permeability on the other hand. As could already be shown for compound **7**, (c) size reduction of the aromatic ring system of **1** is a feasible way to further improve the ligand efficiency of the lead structure. It was known that the introduction of substituents on either of both methylene carbon atoms in α- or β-position of the splitomicin carboxyl function can lead to enhanced activity, thus (d) the introduction of additional substituents on one of the two urea or thiourea nitrogen atoms was envisioned. The combination of these four modifications (a–d) appeared to be especially attractive, because the replacement of the naphthalene moiety by smaller aromatic rings such as benzene and pyridine would compensate for the increase in molar mass associated with the introduction of substituents on N1 and in position 5 and the oxygen sulfur exchange. The fact that only compounds **13e** and **4** but not compounds of type **9** and **10** exhibited very moderate SIRT3 inhibition show, that these scaffolds might be useful starting points to identify sirtuin inhibitors with an advantageous selectivity profile.

5. Conclusion

The formal replacement of the lactone ring in sirtuin inhibitors of the splitomicin type by a stable imidazole-derived heterocyclic ring system enabled the synthesis of novel inhibitors with activity within the low micromolar concentration range. While this activity is superior to the lead compound splitomicin (**1**) it is still not useful as an experimental tool as a chemical probe[29] in the stricter sense of this term. However, the successful identification of a unique inhibitor scaffold adds to the knowledge in the field and complements nicely to the hits identified by a high throughput screening approach[4] performed by the group of Marmorstein and co-workers. Since the ligand efficiency (i.e., potency/size) of the novel compounds is good, decoration of the novel lead structure **6** in position 1 with aryl rings, and positions 5 or 7 with nitro groups and halogen atoms, respectively, seems rewarding. Substitution of N1 with various aromatic substituents would appear to be a logical choice for two reasons. Firstly, SAR investigations from compound class **17** clearly show the beneficial effect upon binding affinity and secondly, the introduction of substituent will diminish the possibility of the formation of tautomers like **6a**. The only tautomers left to form a dynamic equilibrium of types **6b** and **c** were proposed to bind in identical orientations and thus facilitate interpretation of results of biological experiments. From the perspective of a medicinal chemist, compounds with aromatic nitro groups often give rise to toxicity concerns and these metabolically unstable groups should be replaced by more benign or less problematic functional groups with similar electronic and steric properties. Here this is less important, because compounds like **10c** at this stage of investigation will only be regarded as a step towards pharmacological tools or molecular probes and not leads for, for example, anticancer drugs. While lipophilic substitutions are usually the key to increased affinity via non-polar interactions, too many lipophilic substituents often lead to disadvantageous physicochemical characteristics in biological systems. Thus, additional analogs of **6** or compounds from the series **10** with salt-forming basic nitrogen atoms, such as the most active and highly soluble compound **10c** should be prepared and tested. Water soluble hydrochloric acid salts are well suited for crystal soaking experiments in aqueous buffers, and this might aid in the determination of the binding mode of **10c** via X-ray crystallography. The structures reported here consist of a unique sirtuin inhibitor scaffold, thus the novel compounds **10** bridge the gap in chemical space between the labile lead **1**, sirtuin inhibiting indoles[9] like EX-527 (**20**) and recently discovered class of 3-arylidene-indolin-2-one sirtuin inhibitors[30] **21** (Fig. 8). Analogs of **10c** with a single defined binding mode would represent interesting tools for the investigation of molecular interactions and sirtuin functions.

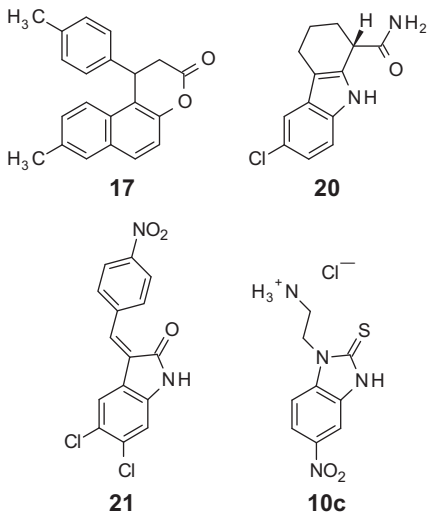


Figure 8. Known classes of sirtuin inhibitors (**17**, EX-527 (**20**), **21**) and the novel sirtuin inhibitors of type **10**.

A combination of SAR data on these related compound classes based on careful exploration of binding modes of these molecules, will facilitate the synthesis of more potent, selective and water soluble sirtuin modulators for pharmacological research.

6. Experimental

6.1. General

NMR spectra were recorded on a Bruker BioSpin UltraShield 400 magnet with Avance III console, a DPX 200 MHz or a JEOL ECLIPSE+ 500 spectrometer, using tetramethylsilane as internal standard. The purity of novel compounds was deduced from NMR data as well as evaluated by HPLC, using a VWR LaChrom or a Shimadzu 20A-Prominence HPLC-system with SPD-M20A photo diode array and ESD-LT II evaporative light scattering detector using CC 125/4 Nucleodur 100-5 C18 ec columns, supplied by Macherey-Nagel. HRMS data were obtained offline on a Micromass Autospec (ESI, methanol (1:1, v/v) infusion at 10 μL/min with polyethylene glycol as reference) or online using a Shimadzu LC–MS-IT-TOF instrument. TLC reaction control was performed on Macherey-Nagel Polygram Sil G/UV254 precoated microplates, spots were visualized under UV-illumination at 254 nm. IR-spectra were recorded on a ThermoFisher Nicolet IR200 FT-IR Spectrometer. Refractive indices were determined using a thermostated Abbé refractometer (Carl Zeiss). Hydrogenations were performed in a HY 1000 apparatus (Hyscho, Bonn). Microwave reactions were executed using a Discover reactor (CEM, Kamp-Lintfort). Docking simulations were generated with MOE (The Molecular Operating Environment) Version 2009.10 from the Chemical Computing Group Inc. with London dG rescoring function and MMFF94× forcefield refinement at default settings.

6.2. General synthetic procedures

For the cyclization of urea derivatives, a solution of the corresponding *o*-phenylene diamine in CH_2Cl_2 is combined with a solution of NaOH in H_2O and stirred. A solution of triphosgene in CH_2Cl_2 is added dropwise slowly into the mixture which is stirred for 12 h. Subsequently the mixture is acidified with HCl (18%). The solvents are evaporated under reduced pressure. The resulting solid is purified as described.

For the cyclization of thiourea derivatives the ethanolic solution of the corresponding *o*-phenylene diamine, triethyl amine and CS_2 are heated to reflux. After the reaction has proceeded for a certain time (as described) the mixture is allowed to cool. The collected crystals are washed as described and dried under reduced pressure.

6.2.1. 1-Isopropyl-5-nitro-1,3-dihydro-2*H*-benzimidazol-2-one (10a)

Compound **10a** was prepared from 0.4 g (2 mmol) N^1-isopropyl-4-nitrobenzene-1,2-diamine in 5 ml CH_2Cl_2, 0.75 g NaOH in 10 ml H_2O, and 0.25 g triphosgene in 5 ml CH_2Cl_2. The resulted solid was dissolved in 50 ml ethyl acetate and washed with brine (2×50 ml) an H_2O (1×50 ml), dried with Na_2SO_4, evaporated under reduced pressure. The resulted crystals were washed with methanol and dried under reduced pressure. Yield: 0.34 g (1.5 mmol), 75%, yellow crystals, mp: 260–261 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 2981; 2835; 2781; 1693; 1626; 1514; 1486; 1366; 1335; 1304; 1185; 1142; 1102; 1069; 1051; 947; 867; 833; 791; 755; 727; 704; 573; 550. ^{1}H NMR: (400 MHz, DMSO-d_6) δ (ppm) = 11.42 (s, 1H, NH); 7.96 (dd, 1H, ^3J_o = 8.8 Hz, ^4J_m = 2.4 Hz, Ar-H); 7.73 (d, 1H, ^4J_m = 2.4 Hz, Ar-H); 7.47 (d, 1H, ^3J_o = 8.8 Hz, Ar-H); 4.63 (sp, 1H, ^3J = 6.8 Hz, C*H*(CH_3)$_2$); 1.45 (d, 6H, ^3J = 6.8 Hz, 2 × CH_3). ^{13}C NMR: (100 MHz, DMSO-d_6) δ (ppm) = 154.0 (C=S); 141.1 (Ar); 134.7 (Ar); 128.4 (Ar); 117.4 (Ar); 108.2 (Ar); 103.8 (Ar); 44.6 (*C*H(CH_3)$_2$); 19.7 (CH_3). HRMS: [*m*/*z*]: calcd [$C_{10}H_{11}N_3O_3$−H]$^-$: 220.0728; found: 220.0729.

6.2.2. 1-Cyclohexyl-5-nitro-1,3-dihydro-2*H*-benzimidazol-2-one (10b)

Compound **10b** was prepared from 0.39 g (1.7 mmol) N^1-isopropyl-4-nitrobenzene-1,2-diamine in 4 ml CH_2Cl_2, 0.55 g NaOH in 4 ml H_2O, and 0.18 g triphosgene in 4 ml CH_2Cl_2. The resulting solid was dissolved in 40 ml ethyl acetate and washed with brine (2×50 ml) and H_2O (1×50 ml), dried with Na_2SO_4, evaporated under reduced pressure. Resulted crystals were washed with methanol and dried under reduced pressure. Yield: 0.37 g (1.4 mmol), 82%, orange crystals, mp: 282 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3017; 2935; 2858; 1599; 1621; 1514; 1487; 1443; 1373; 1336; 1299; 1140; 1107; 1069; 816; 748; 710; 657; 616; 550; 441. ^{1}H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 11.45 (s, 1H, NH); 7.96 (dd, 1H, ^3J_o = 8.8 Hz, ^4J_m = 2.4 Hz, Ar-H); 7.75 (d, 1H, ^4J_m = 2.4 Hz, Ar-H); 7.53 (d, 1H, ^3J_o = 8.8 Hz, Ar-H); 4.28–4.15 (m, 1H, CH); 2.14–1.32 (m, 10H, CH_2 (cyclohexyl)). ^{13}C NMR: (50 MHz, DMSO-d_6) δ (ppm) = 154.0 (C=O); 141.0 (Ar); 134.9 (Ar); 128.4 (Ar); 117.3 (Ar); 108.3 (Ar); 103.7 (Ar); 52.3 (CH); 29.3 (CH_2 (cyclohexyl)); 25.4 (CH_2 (cyclohexyl); 24.6 (CH_2 (cyclohexyl)). HRMS: [*m*/*z*]: calcd [$C_{13}H_{15}N_3O_3$+H]$^+$: 262.1186; found: 262.1196.

6.2.3. 1-(2-Aminoethyl)-5-nitro-1,3-dihydro-2*H*-benzimidazole-2-thione hydrochloride (10c)

Through a solution of 0.25 g (0.74 mmol) *tert*-butyl [2-(5-nitro-2-thioxo-2,3-dihydro-1*H*-benzimidazol-1-yl)ethyl]carbamate in 10 ml dry THF HCl gas is bubbled until precipitation of a solid is complete. Crystals are collected by filtration and washed with THF and dried under reduced pressure. Yield: 0.275 g (0.62 mmol), 83%, black crystals, mp: 229 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3428 cm; 2879 cm; 1614 cm. ^{1}H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 13.54 (s, 1H, NH); 8.28 (s, 3H, NH_3); 8.18 (dd, 1H, *J* = 8.9 Hz, H arom.); 7.96 (d, 1H, *J* = 2.1 Hz, H arom.); 7.83 (d, 1H, *J* = 8.9 Hz, H arom.); 4.57 (t, 2H, *J* = 6.1 Hz, $^2CH_2$); 3.24 (m, 2H, $^1CH_2$). ^{13}C NMR: (50 MHz, DMSO-d_6) δ (ppm) = 171.69; 143.15; 137.39; 130.76; 118.77; 109.18; 105.04; 41.00; 36.74. HRMS: [*m*/*z*]: calcd [M]$^+$: 238.0524; found: 238.0505.

6.2.4. *tert*-Butyl-[3-(5-nitro-2-thioxo-1,3-dihydro-1*H*-benzimidazol-1-yl)propyl]carbamate (10d)

Compound **10d** was prepared from 0.9 g (2.9 mmol) *tert*-butyl-{3-[(2-amino-4-nitrophenyl)amino]propyl}carbamate, 0.5 g CS_2, and 0.6 g triethyl amine in 30 ml of boiling ethanol. After 4 h the reaction was stopped. The collected crystals were washed with ethanol. Yield: 0.85 g (2.4 mmol), 83%, yellow needles, mp: 212 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3194; 2972; 1680; 1536; 1514; 1472; 1436; 1368; 1339; 1323; 1273; 1141; 752; 729; 470. ^{1}H NMR: (400 MHz, DMSO-d_6) δ (ppm) = 13.30 (s, 1H, NH); 8.13 (dd, 1H, ^3J_o = 8.8 Hz, ^4J_m = 2.0 Hz, Ar-H); 7.89 (d, 1H, ^4J_m = 2.0 Hz, Ar-H); 7.60 (d, 1H, ^3J = 8.8 Hz, Ar-H); 6.91 (s, 1H, NH-Boc); 4.27 (t, 2H, ^3J = 7.2 Hz, CH_2); 2.98 (m, 2H, CH_2); 1.84 (m, CH_2); 1.36 (s, 9H, $(CH_3)_3$). ^{13}C NMR: (100 MHz, DMSO-d_6) δ (ppm) = 171.5 (C=S); 155.6 (C=O); 142.9 (Ar); 137.2 (Ar); 130.7 (Ar); 118.6 (Ar); 109.3 (Ar); 104.8 (Ar); 77.6 (*C*(CH_3)$_3$); 41.5 (CH_2); 37.5 (CH_2); 28.2 (CH_3); 27.8 (CH_2). HRMS: [*m*/*z*]: calcd [$C_{15}H_{20}N_4O_3S$+H]$^+$: 353.1278; found: 353.1289.

6.2.5. 1-(3-Aminopropyl)-5-nitro-1,3-dihydro-2*H*-benzimidazole-2-thione hydrochloride (10e)

Through a solution of 0.54 g (1.5 mmol) **10d** in 50 ml dry THF HCl gas is bubbled until precipitation of a solid is complete. Crystals are collected by filtration and washed with THF and dried under reduced pressure. Yield: 0.3 g (1 mmol), 69%, yellow crystals, mp: 235–239 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3043; 2357; 1619; 1514; 1470; 1433; 1336; 1321; 1203; 748; 727; 476. ^{1}H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 13.50 (s, 1H, NH); 8.18 (dd, 1H, ^3J_o = 8.8 Hz, ^4J_m = 2.0 Hz, Ar-H); 8.07 (s, 3H, NH_2·HCl); 7.97 (d, 1H, ^4J_m = 2.0 Hz, Ar-H); 7.76 (d, 1H, ^3J_o = 8.8 Hz, Ar-H); 4.39 (t, 2H, ^3J = 7.4 Hz, CH_2); 2.89 (t, 2H, ^3J = 7.4 Hz, CH_2); 2.05 (qn, 2H, ^3J = 7.4 Hz, CH_2). ^{13}C NMR: (50 MHz, DMSO-d_6) δ (ppm) = 171.6 (C=S); 143.0 (Ar); 137.2 (Ar); 130.8 (Ar); 118.6 (Ar); 109.5 (Ar); 105.0 (Ar); 40.9 (CH_2); 36.3 (CH_2); 25.8 (CH_2). HRMS: [*m*/*z*]: calcd [$C_{10}H_{12}N_4O_2S$−H]$^-$: 251.0608; found: 251.0618.

6.2.6. 1-(2,4-Dimethoxybenzyl)-5-nitro-1,3-dihydro-2*H*-benzimidazol-2-one (10f)

Compound **10f** was prepared from 0.15 g (0.5 mmol) **13e** in 2 ml CH_2Cl_2, 0.4 g NaOH in 2 ml H_2O, and 0.22 g triphosgene in 2 ml CH_2Cl_2. After 5 h the reaction was stopped. The resulting solid was washed with H_2O (2×15 ml) and crystallized from ethanol. Yield: 0.124 g (0.38 mmol), 75%, colorless solid, mp: 224–226 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3123; 3007; 1711; 1617. ^{1}H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 7.44–7.49 (m, 1H, H arom.); 7.42 (t, 1H, *J* = 2.5 Hz NH); 7.06 (d, 1H, *J* = 8.5 Hz, H arom.); 6.59 (d, 1H, *J* = 2.2 Hz, H arom.); 6.47 (dd, 1H, *J* = 2.2/8.4 Hz, H arom.); 6.25–6.37 (m, 2H, H arom.); 5.22 (s, 2H, NH_2); 4.30 (d, 1H, *J* = 5.3 Hz, CH_2); 3.82 (s, 3H, CH_3); 3.74 (s, 3H, CH_3). ^{13}C NMR: (126 MHz, DMSO-d_6) δ (ppm) = 160.24; 157.72; 154.62; 141.31; 135.80; 129.37; 128.37; 117.54; 115.73; 107.70; 104.73; 103.81; 98.35; 55.42; 55.14; one signal covered by DMSO signal. HRMS: [*m*/*z*]: calcd [M]$^+$: 329.1012; found: 329.1019.

6.2.7. 1-(2,4-Dimethoxybenzyl)-5-nitro-1,3-dihydro-2*H*-benzimidazole-2-thione (10g)

Compound **10g** was prepared from 0.152 g (0.5 mmol) **13e**, 0.6 g CS_2, 0.1 g triethyl amine, 20 ml ethanol. After 4 h the reaction was stopped. The mixture was evaporated under reduced pressure and crystallized from ethanol. Yield: 0.137 g (0.39 mmol), 79%, beige solid, mp: 271–274 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3063; 2962; 1617. ^{1}H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 13.41 (s, 1H, NH); 8.10 (dd, 1H, J = 2.3/8.9 Hz, H arom.); 7.92 (d, 1H, J = 2.2 Hz, H arom.); 7.35 (d, 1H, J = 8.9 Hz, H arom.); 6.90 (d, 1H, J = 8.5 Hz, H arom.); 6.61 (d, 1H, J = 2.1 Hz, H arom.); 6.42 (dd, 1H, J = 2.2/8.4 Hz, H arom.); 5.39 (s, 2H, CH_2), 3.86 (s, 3H, CH_3); 3.72 (s, 3H, CH_3). ^{13}C NMR: (126 MHz, DMSO-d_6) δ (ppm) = 172.13; 160.18; 157.59; 142.88; 137.18; 130.61; 128.89; 118.59; 114.98; 109.69; 104.77; 104.75; 98.32; 55.49; 55.13; 41.46. Elemental Anal. Calcd for $C_{16}H_{15}N_3O_4S$: C, 55.65; H, 4.38; N, 12.17. Found: C, 55.70; H, 44.1; N, 11.67. HRM: [m/z]: calcd [M]$^+$: 345.0783; found: 345.0773.

6.2.8. 1-(1,3-Benzodioxol-5-yl)-5-(trifluoromethyl)-1,3-dihydro-2*H*-benzimidazol-2-one (10h)

Compound **10h** was prepared from 0.1 g (0.33 mmol) N^1-1,3-benzodioxol-5-yl-4-(trifluoromethyl)benzen-1,2-diamine in 2 ml CH_2Cl_2, 0.4 g NaOH in 2 ml H_2O, 0.22 g triphosgene in 2 ml CH_2Cl_2. The resulting solid was washed with H_2O (2 × 15 ml) and crystallized from ethanol. Yield: 0.089 g (0.046 mmol), 14%, brown solid, mp: 224–235 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3127; 3042; 2907; 1699; 1625. ^{1}H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 11.47 (s, 1H, NH); 7.30–7.39 (m, 2H, H arom.); 6.95–7.12 (m, 4H, H arom.); 6.14 (s, 2H, CH_2). ^{13}C NMR: (50 MHz, DMSO-d_6) δ (ppm) = 153.47; 147.80; 146.86; 133.55; 128.45; 127.30; 124.59 (q, ^1J(C,F); −272 Hz, CF_3); 122.01 (q, ^2J(C,F); 32 Hz, C5); 120.14; 118.17 (q, ^3J(C,F); 4 Hz, C4); 108.47; 108.16; 107.78; 105.40 (q, ^3J(C,F): 4 Hz, C6); 101.77. HRMS: [m/z]: calcd [M]$^+$: 322.0565; found: 322.0553.

6.2.9. 1,3-Dihydro-2*H*-naphtho[1,2-*d*]imidazol-2-one (4)

A solution of 0.445 g (2 mmol) 4-chloro-2-nitro-1-naphthylamine in 50 ml CH_3OH and 0.06 g Pd/C (10%) are allowed to shake with H_2 (3 bar) in a hydrogenation apparatus until constant pressure was reached. After filtration the product solution is evaporated under reduced pressure. Subsequently 10 ml CH_2Cl_2 and 10 ml NaOH (5 mol/l) and 0.2 g triphosgene is added and the mixture is allowed to stir for 12 h. The mixture is acidified with HCl (18%). The liquid is removed by filtration, resulting in a brown solid. Yield: 0.251 g (1.36 mmol), 68%, mp >360 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3112; 1724; 1662. ^{1}H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 11.48 (s, 1H, NH); 10.90 (s, 1H, NH); 8.08 (d, 1H, J = 8.3 Hz, H arom.); 7.86 (d, 1H, J = 8.4 Hz, H arom.); 7.26–7.57 (m, 4H, H arom.). ^{13}C NMR: (50 MHz, DMSO-d_6) δ (ppm) = 155.22; 128.53; 128.45; 125.61; 124.91; 123.08; 122.96; 120.53; 120.41; 118.99; 110.57.

6.2.10. 5-Nitro-1,3-dihydro-2*H*-naphtho[1,2-*d*]imidazol-2-one (5)

A mixture of 0.184 g 1,3-dihydro-2*H*-naphtho[1,2-*d*]imidazol-2-one, 2 ml glacial acetic acid and 122 μL nitric acid (60%) are heated carefully to 50 °C. After 2.5 h the mixture is poured on ice and washed several times with H_2O, a brown solid is isolated. Yield: 0.146 g, 76%. IR: $\tilde{\nu}$ (cm^{-1}) = 3338; 3011; 2960; 1722; 1596. ^{1}H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 12.28 (s, 1H, NH); 11.37 (s, 1H, NH); 8.61–8.69 (m, 1H, H arom.); 8.20–8.26 (m, 1H, H arom.); 8.17 (s, 1H, H arom.); 7.63–7.72 (m, 2H, H arom.). ^{13}C NMR: (50 MHz, DMSO-d_6) δ (ppm) = 155.34; 138.43; 130.34; 127.58; 126.77; 123.55; 123.13; 121.72; 121.48; 117.95; 110.06.

6.2.11. 1,3-Dihydro-2*H*-naphtho[1,2-*d*]imidazole-2-thione (6)

A solution of 0.223 g (1 mmol) 4-chloro-2-nitro-1-naphthylamine in 50 ml CH_3OH and 0.06 g Pd/C (10%) are allowed to shake with H_2 (3 bar) in a hydrogenation apparatus until constant pressure is reached. After filtration, the product solution 0.65 ml CS_2 and a pellet of NaOH are added, the mixture is heated to reflux until complete reaction (TLC control) is observed. Subsequently the solvent is removed; the resulted solid is washed several times with H_2O. A brown solid is isolated. Yield: 0.251 g, (0.68 mmol) 68%, mp: 362–364 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3058; 2948; 1643. ^{1}H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 13.42 (s, 1H, NH); 12.86 (s, 1H, NH); 8.33 (d, 1H, J = 8.5 Hz, H arom.); 7.86 (d, 1H, J = 8.1 Hz, H arom.); 7.38–7.73 (m, 4H, H arom.). ^{13}C NMR: (50 MHz, DMSO-d_6) δ (ppm) = 166.08; 129.25; 128.59; 128.36; 126.59; 126.56; 124.41; 123.06; 120.80; 119.00; 110.54.

6.2.12. 2-Thioxo-2,3-dihydro-1*H*-benzimidazole-5-carboxylic acid (8)

A mixture of 0.912 g (6 mmol) 3,4-diaminobenzoic acid, 0.24 g NaOH, 1 ml CS_2 and 20 ml H_2O is heated until reflux. Subsequently the free acid is precipitated with glacial acetic acid. The precipitate is washed several times with H_2O and dried under reduced pressure. A brown solid is isolated. Yield: 1.0 g (4.1 mmol), 68%, mp: 362–366 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3065; 2965; 1683; 1629 cm. ^{1}H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 12.80 (s, 3H, NH, COOH); 7.75 (dd, 1H, J = 8.3/1.4 Hz, H arom.); 7.65 (m, 1H, H arom.); 7.20 (d, 1H, J = 8.3 Hz, H arom.). ^{13}C NMR: (50 MHz, DMSO-d_6) δ (ppm) = 134.61; 133.94; 133.42; 129.39; 128.75; 117.43; 116.65; 111.39.

6.2.13. 5-Chloro-1,3-dihydro-2*H*-imidazo[4,5-*b*]pyridin-2-one (9a)

Compound **9a** was prepared from 0.143 g (1 mmol) 6-chloropyridine-2,6-diamine in 2 ml CH_2Cl_2, 0.4 g NaOH in 2 ml H_2O, and 0.22 g triphosgene in 2 ml CH_2Cl_2 in analogy to described[7] procedures. Colorless crystals are obtained from ethanol. Yield: 0.08 g (0.47 mmol), 47%, mp: 374 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3094; 3002; 2817; 1770. ^{1}H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 11.58 (s, 1H, NH); 11.03 (s, 1H, NH); 7.27 (d, 1H, J = 8.0 Hz, H arom.); 7.01 (d, 1H, J = 8.1 Hz, H arom.). ^{13}C NMR: (50 MHz, DMSO-d_6) δ (ppm) = 154.20; 144.34; 139.82; 122.81; 117.08; 115.63.

6.2.14. 5-Chloro-1,3-dihydro-2*H*-imidazo[4,5-*b*]pyridine-2-thione (9b)

A mixture of 0.144 g (1 mmol) 6-chlorpyridine-2,3-diamine, 20 ml ethanol, one pellet of NaOH and 0.5 ml CS_2 is heated to reflux[7] as described. When the reaction is completed (TLC control) the mixture is acidified with HCl (2 mol/l), the solvent is evaporated under reduced pressure. The resulting solid is washed several times with H_2O. Beige solid, yield: 0.125 g (0.67 mmol), 67%, mp: 290–295 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3130; 3063; 2945; 1604. ^{1}H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 13.31 (s, 1H, NH); 12.86 (s, 1H, NH); 7.50 (d, 1H, J = 8.2 Hz, H arom.); 7.19 (d, 1H, J = 8.2 Hz, H arom.). ^{13}C NMR: (50 MHz, DMSO-d_6) δ (ppm) = 170.36; 145.80; 142.48; 124.73; 118.75; 117.43.

6.2.15. 5-Methoxy-1,3-dihydro-2*H*-imidazo[4,5-*b*]pyridine-2-thione (9c)

Compound **9c** was obtained as described[17] using 2,6-dichloropyridine as the starting material in five steps, including methoxylation, nitration, amination, reduction, and cyclization. The overall yield of this sequence was up to 45.7%. The structure of the product was identified by ^{1}H NMR, MS, and IR. A solution of 0.5 g (3 mmol) 6-methoxy-3-nitropyridine-2-amine 100 ml CH_3OH, 2 ml H_2O, and 0.1 g Pd/C (10%) are allowed to shake with H_2 (3 bar) in a hydrogenation apparatus until constant pressure. After filtration, the product solution is heated with 1 ml CS_2 and 0.225 g KOH until reflux. When the reaction is completed (TLC control) the solvent is evaporated under reduced pressure. The resulted solid is recrystallized

from ethanol. Yield: 0.187 g (1.1 mmol), 35%, mp: 223–225 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3262; 3051; 1624. ^1H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 12.99 (s, 1H, NH); 12.54 (s, 1H, NH); 7.43–7.47 (d, 1H, J = 8.5 Hz, H arom.); 6.55–6.60 (d, 1H, J = 8.5 Hz, H arom.); 3.83 (s, 3H, CH_3). ^{13}C NMR: (50 MHz, DMSO-d_6) δ (ppm) = 167.60; 160.12; 143.62; 120.17; 119.65; 103.90; 53.49.

6.2.16. *N*-(2,4-Dinitrophenyl)-1,3-benzodioxol-5-amine (12e)

Compound **12e** was obtained by heating a mixture of 1.709 g (7 mmol) 2,4-dimethoxybenzylamine and 5 mmol 2,4-dinitrofluorobenzene, 10 mmol $NaHCO_3$, 50 ml acetone, and 5 ml H_2O to reflux for 1–2 h. The solvent is evaporated under reduced pressure. The resulted solid is washed with H_2O and recrystallized from ethanol. Yield: 1.37 g (4.1 mmol), 82%, yellow needles, mp: 107–109 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3352; 3112; 1624. ^1H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 9.18 (t, 1H, J = 6.0 Hz, NH); 8.85 (d, 1H, J = 2.7 Hz, H arom.) 8.22 (dd, 1H, J = 2.7/9.6 Hz, H arom.); 7.06–7.18 (m, 2H, H arom.); 6.61 (d, 1H, J = 2.3 Hz, H arom.); 6.47 (dd, 1H, J = 2.3/8.3 Hz, H arom.); 4.59 (d, 2H, J = 6.0 Hz, CH_2); 3.85 (s, 3H, CH_3); 3.73 (s, 3H, CH_3). ^{13}C NMR: (126 MHz, DMSO-d_6) δ (ppm) = 160.17; 157.78; 147.95; 134.83; 129.88; 129.79; 128.75; 123.49; 116.39; 115.31; 104.65; 98.50; 55.49; 55.14; 41.44. HRMS: [m/z]: calcd $[M]^+$: 333.0951; found: 333.0951.

6.2.17. N^1-(2,4-Dimethoxybenzyl)-4-nitro-benzene-1,2-diamine (13e)

To a boiling solution of 0.833 g (2.5 mmol) **12e** in 90 ml methanol a solution of 2.1 g $NaHCO_3$ and 6.1 g $Na_2S{\cdot}9H_2O$ in 25 ml H_2O is dropped over 1 h. Subsequently the mixture is allowed to boil at reflux for 4 h. When the reaction is finished, the mixture is poured in 300 ml cold H_2O. The resulted crystals are washed with H_2O and recrystallized from ethanol. Yield: 1.37 g (2.1 mmol), 82% red crystals, mp: 160–162 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3390; 3328; 1644. ^1H NMR: (200 MHz, DMSO-d_6) δ (ppm) = 7.44–7.49 (m, 1H, H arom.); 7.42 (t, 1H, J = 2.5 Hz, NH); 7.06 (d, 1H, J = 8.5 Hz, H arom.); 6.59 (d, 1H, J = 2.2 Hz, H arom.); 6.47 (dd, 1H, J = 2.2/8.4 Hz, H arom.); 6.25–6.37 (m, 2H, H arom.); 5.22 (s, 2H, NH_2); 4.30 (d, 1H, J = 5.3 Hz, CH_2); 3.82 (s, 3H, CH_3); 3.74 (s, 3H, CH_3). ^{13}C NMR: (126 MHz, DMSO-d_6) δ (ppm) = 159.85; 157.90; 142.38; 136.65; 134.53; 128.79; 117.87; 115.63; 107.28; 107.24; 104.50; 98.37; 55.42; 55.15; 40.98. Elemental Anal. Calcd for $C_{15}H_{17}N_3O_4$: C, 59.40; H, 5.65; N, 13.85. Found: C, 59.41; H, 5.72; N, 13.50.

6.2.18. 2-Nitro-*N*-[4-(trifluoromethyl)-phenyl]-1,3-benzodioxol-5-amine (15)

A mixture of 1.646 g (12 mmol) 3,4-(methylenedioxy)aniline and 10 mmol 1-fluoro-2-nitro-4-(trifluoromethyl)benzene, 20 mmol $NaHCO_3$, 100 ml acetone, and 10 ml H_2O is heated until reflux for 1–2 h. The solvent is evaporated under reduced pressure. The resulted solid is washed with H_2O and recrystallized from ethanol. Yield: 1.625 g (0.67 mmol), 67%, red solid, mp: 172–175 °C. IR: $\tilde{\nu}$ (cm^{-1}) = 3350; 1583. ^1H NMR: (500 MHz, DMSO-d_6) δ (ppm) = 10.07 (s, 1H, NH); 8.88 (d, 1H, J = 2.8 Hz, H arom.); 8.21 (dd, 1H, J = 2.8/9.6 Hz, H arom.); 6.85 (dd, 1H, J = 1.6/8.3 Hz, H arom.); 7.00–7.10 (m, 2H, H arom.); 6.98 (d, 1H, J = 1.8 Hz, H arom.); 6.85 (m, 1H, H arom.); 6.11 (s, 2H, CH_2). ^{13}C NMR: (126 MHz, DMSO-d_6) δ (ppm) = 148.72; 148.01; 147.01; 136.53; 131.97; 131.40; 130.16; 123.79; 120.21; 117.54; 109.51; 108.36; 102.27. Elemental Anal. Calcd for $C_{13}H_9N_3O_6$: C, 51.49; H, 2.99; N, 13.86. Found: C, 51.16; H, 3.23; N, 13.71.

6.3. Recombinant proteins

Human Sirt1 was expressed in *E. coli* as an N-terminally GST-tagged, human Sirt2 as an N-terminally His_6-tagged and human Sirt3 as an C-terminally His_6-tagged fusion protein. The enzymes were purified as described[31] with minor modifications. Purity and identity of the produced enzymes were verified using SDS gel electrophoresis. The deacetylase activity of the sirtuins was dependent on the sirtuin cofactor NAD^+ and could be inhibited with the endogenous sirtuin inhibitor nicotinamide.

6.4. Fluorescent deacetylase assay

Rates of inhibition of recombinant human SIRT1, SIRT2, and SIRT3 were determined using a homogeneous fluorescent deacetylase assay[32] carried out in 96-well plates. The assay mixture contained the fluorescent deacetylase substrate ZMAL (10.5 μM), NAD^+ (500 μM), the inhibitors at various concentrations and DMSO (5–10% v/v). Total assay volume was 60 μL. The amount of enzyme preparation was dependent on the activity of the preparation that was used and varied from batch to batch. To assure initial state conditions we usually adjusted to 10–30% substrate conversion without inhibitor. A mixture solely with DMSO was used as a control. After incubation (4 h, 37 °C) a stop solution (60 μL) was added containing trypsin (1 mg × ml^{-1}, from bovine or porcine pancreas, 10,000–200,000 BAEE units × mg^{-1}) and nicotinamide (8 mM) to cleave off the fluorescent aminocoumarin. Fluorescence intensity was measured with a microplate reader (BMG Polarstar, λ_{ex} = 390 nm, λ_{em} = 460 nm). Comparison of the fluorescence intensity of the DMSO control with a probe containing a potential inhibitor was used to determine the rate of inhibition. All inhibition determinations were carried out at least in duplicates. IC_{50} values were determined using GraphPad Prism software.

Acknowledgment

The authors acknowledge financial support by DFG Grants Li765/4-2 and Ju295/8-1.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.01.026. These data include MOL files and InChiKeys of the most important compounds described in this article.

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