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Brief Article

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Novel 3-Arylideneindolin-2-ones as Inhibitors of NAD$^+$-Dependent Histone Deacetylases (Sirtuins)

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Class III histone deacetylases (sirtuins) play pivotal roles in many cellular processes. They are linked to extended lifespan and to the pathogenesis of cancer and neuronal disorders. We present novel sirtuin inhibitors based on a 6,7-dichloro-2-oxindole scaffold with low micromolar activity. In vitro activity was rationalized by docking studies, and hyperacetylation of sirtuin targets could be demonstrated in cell culture.

Introduction

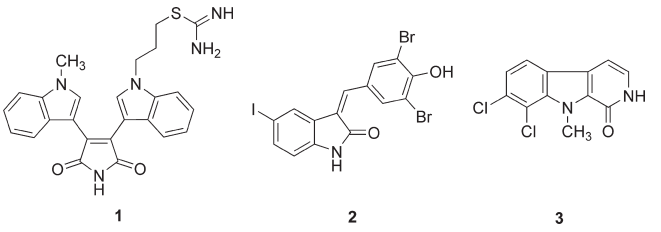
Reversible acetylation constitutes a key role in the regulation of cellular processes by modulating the activity of numerous important proteins such as histones and p53. Class III histone deacetylases (sirtuins) cleave acetyl groups from lysines in histones and other proteins, affecting metabolism and apoptosis. Sirtuins require nicotinamide adenine dinucleotide (NAD$^+$ [a]) as their cofactor for catalysis.

Seven human subtypes of sirtuins (SIRT1−7) have been identified.[1] SIRT1 deacetylates proteins such as p53 or BCL6, its expression being induced by calorie restriction (CR). SIRT1 alongside with SIRT3 promotes an extended lifespan in various organisms.[2] SIRT2 deacetylates tubulin[1] and its inhibition results in neuroprotection,[3−5] while elevated levels of SIRT3 have been detected in breast cancer.[6] Thus, sirtuin inhibitors are highly interesting as biochemical tools and potential drugs. Important sirtuin inhibitors are sirtinol,[7] splitomicins,[8,9] cambinol, and other thioureas.[10,11] We also reported that the adenosine mimetics bisindolylmaleimide Ro-31-8220 (**1**) and oxindole GW5074 (**2**) (Chart 1) inhibit sirtuins, but only **1** exhibited cellular hyperacetylation.[12] Following our concept of combining structural elements of bioactive natural compounds,[13] we synthesized analogues of **2** with an oxindole scaffold containing the distinctive 6,7-dichloroindole moiety of the alkaloid bauerine C (**3**). β-Carboline **3** was originally isolated from the blue-green alga *Dichotrix baueriana* and antiproliferative as well as antiviral activity has been reported.[14] We have already prepared hybrids between **3**[15] and ruteacarpine with increased cytotoxicity.[13] Here, we similarly combine structural elements of alkaloid **3** with those of the sirtuin inhibitor **2**.

Results and Discussion

Chemistry. 6,7-Dichloro-2-oxindole (**15**) was prepared from 2,3-dichloroaniline (**16**) and ethyl (methylthio)acetate (**17**) in two steps (Scheme 1) using a modified Gassman synthesis.[16] The intermediate thioacetate **18** was not isolated but was present as seen by MS analysis. Reductive desulfurization of the 3-methylthioindolinone **19** with Raney nickel gave the 6,7-dichlorinated indolinone **15**, with no dehalogenation observed.[17] Oxindole **15** was then reacted with several aromatic aldehydes using piperidine as a base[18] to attain the arylidene products **4**−**14**. Because of the exocyclic double bond, the 3-arylideneindolinones may exist as the *E* or *Z* isomers or as mixture of both. NOE experiments as previously described in the literature[18] were employed to elucidate this (see Supporting Information), the results of which indicated that inseparable *E*/*Z* mixtures were mostly obtained. *E*/*Z* ratios were determined using the corresponding integrals in the ^{1}H NMR spectra and were shown not to change over time (Table S1). The 3,5-dimethylpyrrole derivative **10** and the two pyridines **11** and **12**, however, were obtained as single isomers because of intramolecular interactions that favor one isomer[18] (see Supporting Information).

Chart 1. Lead Structures



Enzyme Inhibition. All compounds were screened against SIRT1, -2, and -3 in a fluorescence assay,[19] with IC_{50} determined for the best inhibitors (Table 1). The lead compound **2** and the kinase inhibitor sunitinib[20] were tested as structurally similar compounds. In contrast to **2** sunitinib did not show good sirtuin inhibition. Dibromophenol derivative **4** exhibited activity against SIRT1 comparable to **2**, which was more selective toward SIRT2 and -3, suggesting a beneficial effect of the two chlorine substituents. Removal of the two bromides in **5** led to a decrease in activity toward sirtuins, while no inhibition was seen with the 3,4,5-trimethoxy derivative **6**. The *p*-fluorobenzylidene substituted oxindole

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[a] Abbreviations: NAD$^+$, nicotinamide adenine dinucleotide; Sir2, silent information regulator 2.

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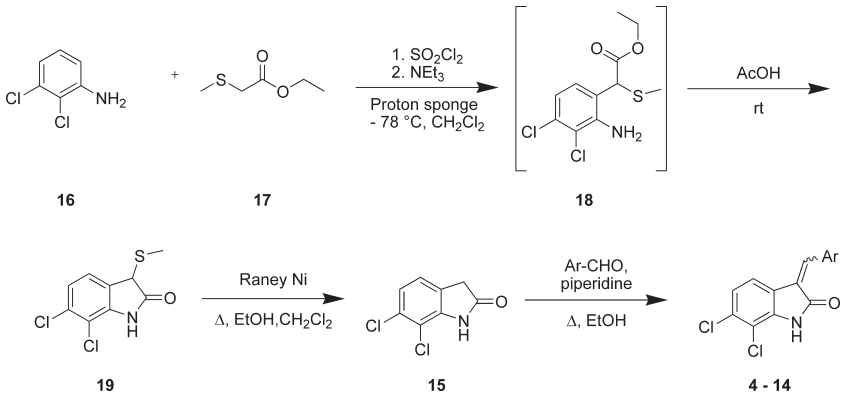
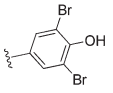
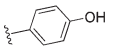
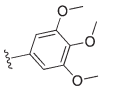
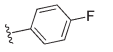
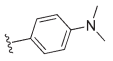
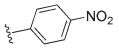
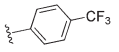
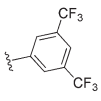
Scheme 1. Synthesis of Compounds **4**−**14** via the 6,7-Dichlorooxindole **15**[a]



[a] See Table 1 for definition of Ar.

Table 1. Sirtuin Inhibition

		IC_{50}±SE [μM] or inhibition [%] @ 50 μM		
Compound	Ar	SIRT1	SIRT2	SIRT3
2	-	41.6 ± 0.7	15.6 ± 1	25.1 ± 0.6
3	-	NA[a]	87.2 ± 9.5	NA
Sunitinib	-	NA	122.7 ± 23.6	26.4%
4		40.6 ± 0.6	28.4 ± 2.5	23.6 ± 0.4
5		NA	34.7%	13.5%
6		NA	NA	NA
7		24.4%	33.4 ± 5.6	NA
8		20.7%	NA	NA
9		30.2%	10.4 ± 1.4	NA
10		19.9%	135.4 ± 14.8	NA
11		22.7%	20.6%	NA
12		19.6%	16.5%	NA
13		45.4%	18.3%	NA
14		19.8%	10.1%	NA

[a] Inhibition of < 10% at 50 μM.

7 inhibited SIRT2 (IC_{50} = 33.4 μM) but not the other isotypes. Heterocyclic analogues **10**, **11**, and **12** only displayed weak inhibition of SIRT1 and SIRT2 and did not inhibit SIRT3. The most potent SIRT2 inhibitor proved to be *p*-nitro compound **9**, with its IC_{50} of approximately 10 μM exceeding model compound **2** and its analog **4**. Additional derivatives with electron withdrawing groups (**13** and **14**) were less potent against SIRT2 in comparison to **9** but showed increased activity against SIRT1 in the case of the *p*-trifluoromethyl compound **13**. Thus, SIRT2 binding affinity is not generally affected by an electron deficient phenyl ring but might depend on distinct interactions. Our results indicate that 3-arylideneindolinones are able to exert significant inhibition on sirtuins with a preference for SIRT2.

Western Blot Analysis. To validate sirtuin inhibition in cells, **7** and **4** were incubated with MCF-7 breast adenocarcinoma cells for 6 and 16 h, respectively, and tested for tubulin hyperacetylation which depends on SIRT2.[21] Because of solubility problems, **9**, the most potent inhibitor in vitro, could not be evaluated in cell culture. Trichostatin (TSA), which inhibits the tubulin deacetylase HDAC6, and the sirtuin inhibitor Ro-31-8220 (**1**) were used as reference compounds. In contrast to lead compound **2**, hyperacetylation could be observed for **4** and, to a lesser extent, **7** after 6 h (Figure 1). After 16 h a small degree of hyperacetylation could also be detected for **2** (data not shown). As reported in previous work,[12] HDAC6 (inhibition by TSA) contributes to a much greater extent to tubulin hyperacetylation than SIRT2 (inhibition by **1**).

Cell-Based ELISA Assay. A cell-based ELISA-type assay[22] was also performed to validate initial results. In Hep G2 cells **4** induced α-tubulin hyperacetylation, while the *p*-fluorobenzylidene derivative **7** only displayed weak effects due to poor solubility in the growth medium (see Supporting Information, Figure S6). Protein content was measured for normalization and did not show any significant cytotoxic effects (data not shown).

Docking Study. A docking study was carried out using the human SIRT2 X-ray structure (PDB code 1J8F) and the GOLD software to rationalize our findings as in previous work.[9] The docking results clearly indicate that only the *Z* isomers are able to interact with the nicotinamide binding pocket (pocket C) of SIRT2 (Figure 2A and Figure S6). In contrast, docking of the *E* isomer did not result in an energetically favorable binding mode. Thus, lead compound **2** and the other indolinones cannot bind to the adenosine pocket independent of the *E*/*Z* configuration, in correspondence with competition data where increasing NAD^+ concentrations did not diminish inhibition by **2** (data not shown). An overlay of our most potent inhibitor **9** and cambinol shows that the lactam NH of the indolinone forms hydrogen bonds with the backbone carbonyl of Gln167 and

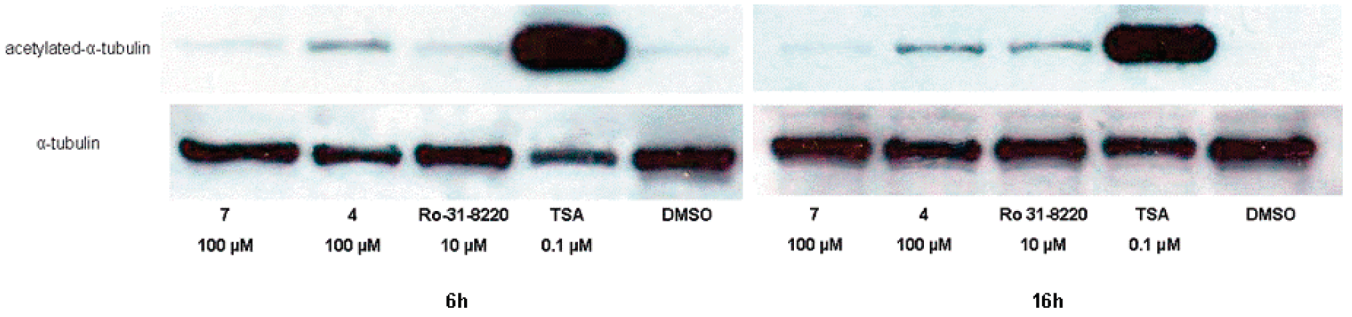


Figure 1. Western blot analysis of **7** and **4** after 6 h (left) and 16 h (right). Hyperacetylation of tubulin is already visable after 6 h and increases over time (16 h). The sirtuin inhibitor Ro-31-8220 and the HDAC inhibitor trichostatin A (TSA) were used as reference inhibitors.

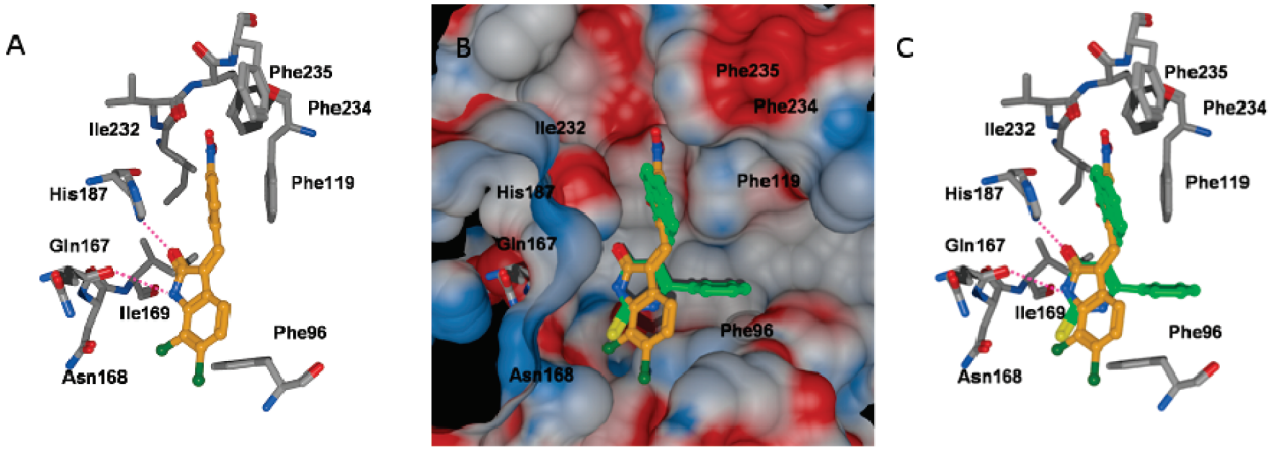


Figure 2. (A) Docking solution of **9** (orange) binding to the C pocket of human SIRT2 (PDB code 1J8F). Only the *Z*-isomer is able to bind in such a way allowing the *p*-nitrophenyl substituent to protrude into the hydrophobic lysine tunnel and interact with apolar residues. (B, C) Comparison of the docking solution of **9** (orange) and cambinol (green). The amide moiety of each compound binds to His187 and the backbone CO of Gln 167. The overlay reveals additional space for substituents in position 4 of the oxindole. (B) The molecular surface of SIRT2 is shown and is colored according to the electrostatic potential (red, negative; blue, positive).

His187 as observed for cambinol (Figure 2B,C). This indicates that additional substituents could be introduced at position 4 of the oxindole to mimic the phenyl ring of cambinol. Compounds bearing more sterically demanding groups at the benzylidene residue (e.g., **6**, **8**, **10**) could not be docked in the conformation observed for **9** because of steric clashes with the residues of the narrow acetyl−lysine channel (Phe119, His187, Ile232, Phe235).

Conclusion

A convenient approach toward the synthesis of novel SIRT inhibitors with a 6,7-dichloro-substituted oxindole scaffold was developed by means of a modified Gassman synthesis. Docking-based SAR studies using human SIRT2 X-ray structure suggest that the 3-arylideneindolinones do not bind to the adenosine pocket A but to the C subpocket in a way similar to cambinol, a known inhibitor of sirtuins. Compound **4** exhibits good activity against SIRT1 and -2, while **9** is a selective SIRT2 inhibitor. Western blots confirmed hyperacetylation of the SIRT2 target α-tubulin due to sirtuin inhibition by **4** in cell culture. Thus, we were able to improve selectivity and cellular activity in comparison to the lead inhibitor **2**. Furthermore, the neuroprotective effects that have been reported for some 3-substituted oxindoles[23,24] suggest a link to SIRT2 inhibition, but additional investigations would be needed for clarification.

Work is in progress to further enhance the affinity and selectivity of this new class of sirtuin inhibitors.

Experimental Section

General Information. For details about chemicals and equipment, see Supporting Information.

General Procedure for the Synthesis of 3-Arylideneindolin-2-ones 4−14. Amounts of 1 equiv of 6,7-dichloroindolin-2-one (**15**) (see Supporting Information), 1.2 equiv of the appropriate aldehyde, and 0.1 equiv of piperidine were dissolved in 30 mL of ethanol and heated to reflux for 3 h. After the mixture was cooled, the precipitate was filtered off and recrystallized from the given solvent.

(*E*/*Z*)-6,7-Dichloro-3-(3,5-dibromo-4-hydroxybenzylidene)-indolin-2-one (4). Amounts of 278 mg (1.38 mmol) of **15**, 385 mg (1.38 mmol) of 3,5-dibromo-4-hydroxybenzaldehyde, and 0.01 mL piperidine were reacted as given above. Yield: 381 mg (60%) as yellow crystals from ethanol/acetic acid (1:1). Mp 315 °C; ^{1}H NMR (500 MHz, DMSO-d_6, TMS) δ 11.27 (br s, 0.7 × 1 H, N−H, *Z*), 11.24 (br s, 0.3 × 1 H, N−H, *E*), 10.81 (br s, 1 H), 8.78 (s, 0.7 × 2 H, 2″-/6″-H, *Z*), 7.92 (s, 0.3 × 2 H, 2″-/6″-H, *E*), 7.81 (s, 0.7 × 1 H, vinyl-H, *Z*), 7.63 (d, J = 8.2 Hz, 0.7 × 1 H, 4-H, *Z*), 7.61 (s, 0.3 × 1 H, vinyl-H, *E*), 7.42 (d, J = 8.2 Hz, 0.3 × 1 H, 4-H, *E*), 7.26 (d, J = 8.2 Hz, 0.7 × 1 H, 5-H, *Z*), 7.18 (d, J = 8.2 Hz, 0.3 × 1 H, 5-H, *E*); *E*/*Z* ratio (%) 30:70; MS EI *m*/*z* (relative intensity, %) 467 [M$^{+\bullet}$] (32), 465 [M$^{+\bullet}$] (88), 463 [M$^{+\bullet}$] (100); HRMS calcd, 460.8221; found, 460.8203; IR ν_{max} (cm^{-1}) 3068 (NH), 1604 (C=O). Anal. ($C_{15}H_7Br_2Cl_2NO_2$) C, H, N.

(*E*/*Z*)-6,7-Dichloro-3-(4-nitrobenzylidene)indolin-2-one (9). Amounts of 250 mg (1.24 mmol) of **15**, 224 mg (1.48 mmol) of 4-nitrobenzaldehyde, and 0.01 mL of piperidine were reacted as given above. Yield: 300 mg (72%) as an orange solid from ethanol. Mp 340 °C; ^{1}H NMR (600 MHz, DMSO-d_6, TMS) δ 11.39 (br s, 0.43 × 1 H, N−H, *Z*), 11.38 (br s, 0.57 × 1 H, N−H, *E*), 8.48

(d, J = 8.7 Hz, 0.43 × 2 H, 3″-/5″-H, *Z*), 8.36 (d, J = 8.7 Hz, 0.57 × 2 H, 3″-/5″-H, *E*), 8.31 (d, J = 8.7 Hz, 0.43 × 2 H, 2″-/6″-H, *Z*), 8.06 (s, 0.43 × 1 H, vinyl-H, *Z*), 7.95 (d, J = 8.7 Hz, 0.57 × 2 H, 2″-/6″-H, *E*), 7.81 (s, 0.57 × 1 H, vinyl-H, *E*), 7.76 (d, J = 8.2 Hz, 0.43 × 1 H, 4-H, *Z*), 7.33 (d, J = 8.2 Hz, 0.57 × 1 H, 4-H, *E*), 7.30 (d, J = 8.2 Hz, 0.43 × 1 H, 5-H, *Z*), 7.11 (d, J = 8.2 Hz, 0.57 × 1 H, 4-H, *E*); *E*/*Z* ratio (%) 57:43; MS EI *m*/*z* (relative intensity, %) 338 [$M^{+\bullet}$] (12), 336 [$M^{+\bullet}$] (63), 334 [$M^{+\bullet}$] (100); IR ν_{max} (cm^{-1}) 3427 (NH), 1711, 1608 (C═O), 1516, 1342 (NO_2). Anal. ($C_{15}H_8Cl_2N_2O_3$) C, H, N.

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Supporting Information Available: Procedures and full experimental data for all synthesized compounds, NMR spectra from NOE experiments, and biochemical assay procedures and docking studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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