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Carprofen Analogues as Sirtuin Inhibitors: Enzyme and Cellular Studies

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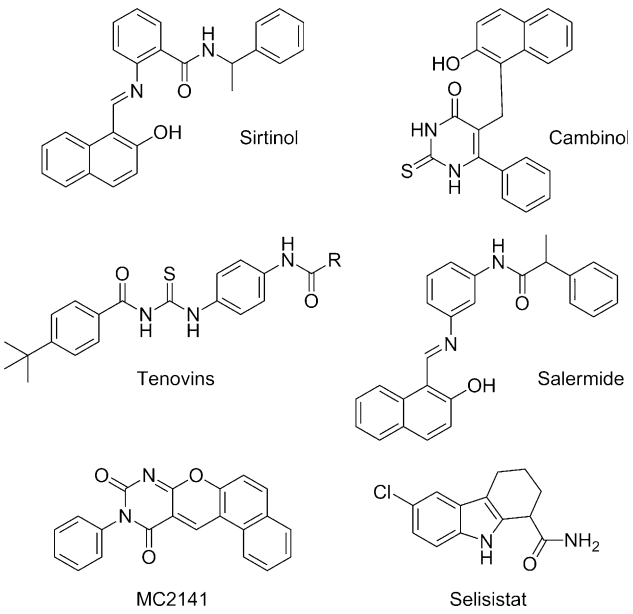
Among the histone deacetylase (HDAC) family, class III HDACs, also named sirtuins (SIRT1–7),[1] are characterized by a conserved 270-amino-acid catalytic core domain, and catalyze the removal of an acetyl moiety from the ε-amino group of lysine residues through a NAD^+-dependent deacetylation mechanism.[2] In addition to histones (H1, H3, and H4), SIRTs can deacetylate a variety of nonhistone substrates (e.g., FoxO, NF-κB, p53, p73, p300, α-tubulin, etc.), thus playing critical roles in different biological processes including control of gene expression, metabolism, and aging.[3,4] In particular, deregulation of SIRT1 is involved in many human processes or diseases, such as obesity, inflammation,[5,6] muscle differentiation,[7] and neurodegeneration.[8]

In cancer, the role of SIRT1 is highly debated. Indeed, on one hand, SIRT1 was found to be upregulated in many malignancies, such as human prostate cancer, acute myeloid leukemia, nonmelanoma skin cancer, primary colon cancer, and breast cancer.[9] In addition, SIRT1 might promote tumorigenesis as a result of the negative regulation of the tumor suppressor proteins p53, hypermethylated in cancer 1 (HIC1), and deleted in breast cancer 1 (DBC1).[9] On the other hand, SIRT1 expression was found to be reduced in glioblastoma, bladder carcinoma, prostate carcinoma, and ovarian cancer, when compared to the corresponding normal tissues,[10] and overexpression of SIRT1 in $APC^{min/+}$ mice showed a protective role in colon cancer.[11] Thus, depending on the experimental model and the tumor type under study, it seems that SIRT1 might be involved in tumor promoter or tumor suppressor functions.[12]

Despite SIRT2 having been initially considered less involved than SIRT1 in cancer development due to its mainly cytosolic localization and its ability to deacetylate α-tubulin, its role in the pathogenesis and development of cancer has been recently suggested.[13,14] Thus, dual inhibition of SIRT1 and SIRT2 is now deemed necessary to obtain efficient antitumor effect.[15]

To date, a few SIRT1/2 inhibitors have been validated in cancer. Sirtinol was reported to induce senescence-like growth arrest in human breast cancer (MCF-7) cells and lung cancer



(H1299) cells,[16,17] and very recently, it has been shown to modulate androgen-, estrogen-, and insulin-like growth factor-1-mediated pathways in LNCaP cells, with effects not due solely to sirtuin inhibition.[18] Cambinol induces apoptosis in BCL6-expressing Burkitt lymphoma cells,[19] salermide shows tumor-specific apoptosis in a wide range of human cancer cell lines,[20] tenovins decrease tumor growth in vivo as single agents at low micromolar concentrations,[21] and MC2141 displays high antiproliferative activity against Raji, DLD1, and HeLa cells.[22] In contrast, SIRT1-selective inhibitor selisistat (EX-527, SEN0014196)[23] efficiently increases p53 acetylation in cells without reported effects on cell growth, viability, or p53-controlled gene expression,[24] and it has entered phase II clinical trials for the treatment of Huntington's disease.[25]

Nonsteroidal anti-inflammatory drug (NSAID) carprofen was shown to activate the p38 mitogen-activated protein kinase (MAPK) pathway leading to an increased level of $p75^{NTR}$ protein and induction of apoptosis in prostate (PC-3 and DU-145) and

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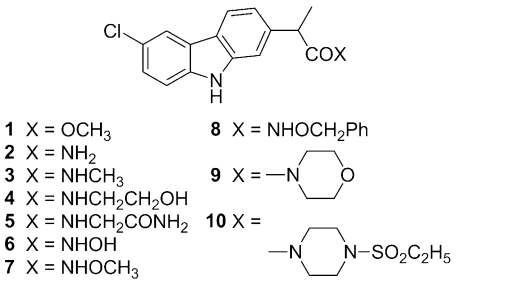
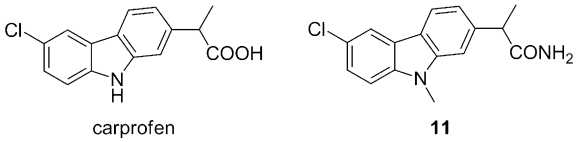
bladder (T24) cancer cells.[26] In this assay, carprofen was one order of magnitude more potent than a series of related profens. Due to the structural similarity between carprofen and selisistat, we prepared a small library of carprofen-related derivatives (**1**–**11**) by retaining the carbazole core and replacing the carboxylic acid function with an ester, (substituted)amide, or (substituted)hydroxamate group. Compounds **1**–**11** were

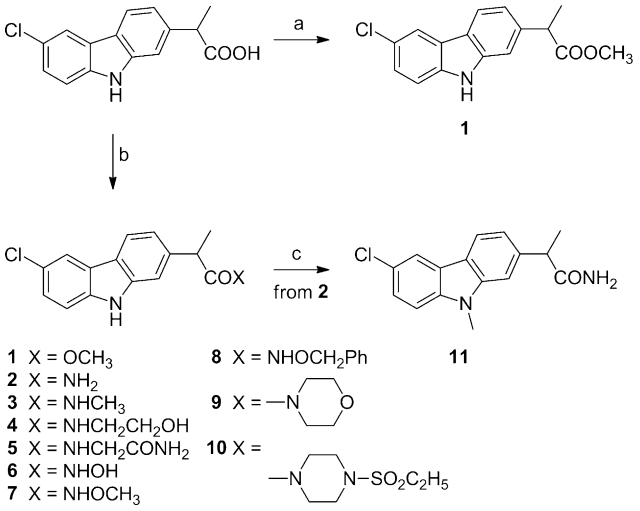




tested against SIRT1 and SIRT2, and among them, compound **2** was selected for evaluation of p53 and α-tubulin acetylation levels, in the p53 wild-type breast cancer MCF-7 cells and human leukemia U937 cells, respectively. Moreover, the effects of 6-chloro-9*H*-carbazol-2-ylamides **2**, **4**, **6**, **9**, and **11** on cell cycle, apoptosis, and granulocytic differentiation in U937 cells was explored.

The synthetic pathway followed for the preparation of **1**–**11** is depicted in Scheme 1. Methyl ester **1** was prepared by reacting carprofen, methanol, and 96% sulfuric acid at reflux for five hours according to a literature procedure.[27] Reaction of carprofen with thionyl chloride led to the formation of the corresponding acid chloride, which was treated with appropriate amines to afford carboxamides **2**–**10**. Finally, use of iodomethane in the presence of potassium carbonate in dry *N*,*N*-dimethylformamide (DMF) converted **2** into the related *N*-methylcarbazole derivative (**11**).

Carprofen and its analogues (**1**–**11**) were tested in vitro against human recombinant (hr) SIRT1 and SIRT2 at a concentration of 50 μM using a fluorescent biochemical assay.[21] Since the carprofen-based compounds possess a chiral center, enantiomers of 2-(6-chloro-9*H*-carbazol-2-yl)propanamide **2** and its *N*-methylpropanamide derivative **3** were isolated on a semipreparative scale by enantioselective HPLC (for details, see the Supporting Information), and the individual enantiomers were subjected to biological investigation. As shown in Table 1, carprofen and its methyl ester **1** showed weak SIRT1 inhibitory effects. Among the 6-chloro-9*H*-carbazol-2-ylamides (**2**–**11**), the most potent SIRT1 inhibition activities were observed with primary propanamide **2** (with no differences between the two enantiomers) and its 9-methyl analogue **11**, with 78.3 and



Scheme 1. *Reagents and conditions*: a) CH_3OH, H_2SO_4, reflux, 5 h; b) 1) $SOCl_2$, reflux, 4 h, 2) XNH_2 or XNH_2·HCl/Et_3N, CH_3CN (CH_3CN/CH_3OH for **6**–**8**), RT, 3 h→o/n; c) CH_3I, K_2CO_3, DMF, RT, 24 h.

Table 1. Inhibitory activities of carprofen and derivatives **1**–**11** at 50 μM against hrSIRT1 and hrSIRT2.

Compd	Inhibition[a] [%]	
	SIRT1	SIRT2
1	37.2 ± 1.5	NI
2	78.3 ± 3.9	59.6 ± 2.4
(+)-**2**	79.0 ± 2.8	61.7 ± 1.8
(−)-**2**	77.5 ± 3.9	59.1 ± 1.5
3	NI	34.2 ± 1.7
(+)-**3**	NI	23.3 ± 1.2
(−)-**3**	NI	34.7 ± 1.7
4	36.0 ± 1.8	9.8 ± 0.4
5	21.5 ± 0.9	30.5 ± 1.5
6	50.0 ± 2.6	21.0 ± 0.8
7	19.2 ± 1.0	39.3 ± 2.0
8	NI	54.4 ± 2.2
9	56.4 ± 2.8	6.0 ± 0.3
10	14.0 ± 0.7	21.2 ± 1.1
11	59.5 ± 3.0	33.9 ± 1.7
Carprofen	20.2 ± 1.0	13.8 ± 0.4
Selisistat	83.6 ± 4.2	45.5 ± 2.8

[a] Data represent the mean ± SD of at least two experiments. No inhibition (NI) observed at the dose evaluated.

59.5% inhibition at the tested dose, respectively. The IC_{50} values for compounds **2** and **11** against SIRT1 were determined to be 7.1 and 38.0 μM, respectively (Table 2). Hydroxamic acid **6** was active (50% SIRT1 inhibition at 50 μM), but the inhibitory action was lost when the oxygen atom was substituted to give a hydroxamate (*O*-methyl: **7**; *O*-benzyl: **8**). The introduction of substituents other than hydrogen at the primary amide function of **2** gave rise to analogues with moderate to no SIRT1 inhibition—compound **4**, bearing a 2-hydroxyethyl group, displayed 36% inhibition at 50 μM, while *N*-methylpropanamide **3** was totally inactive). Insertion of cyclic amines, such as morpholine (**9**), and *N*-ethylsulfonylpiperazine (**10**), at the carpro-

Table 2. Inhibitory data for compounds **2** and **11** against SIRT1 and SIRT2.

Compd	IC_{50}[a] [μM]	
	SIRT1	SIRT2
2	7.1 ± 0.4	64.9 ± 5.8
11	38.0 ± 1.9	99.7 ± 8.8

[a] Data represent the mean ± SD of two experiments.

fen scaffold gave rise to appreciable (**9**: >50%) or low (**10**: 14%) SIRT1 inhibition.

Against SIRT2, compound **2** displayed the highest inhibitory activity (59.6% inhibition at 50 μM; IC_{50} = 64.9 μM; no stereoselectivity), followed by *O*-benzyl hydroxamate **8** (54.4% inhibition). *O*-Methyl hydroxamate **8**, *N*-methyl derivatives **3** and **11**, and *N*-glicinamide **5** all exhibited greater than 30% inhibition of SIRT2, while the remaining compounds displayed only very low SIRT2 inhibitory activities at 50 μM.

SIRT1/2 inhibition by compound **2** was confirmed by functional assays through Western blot analyses. The acetylation levels of p53 (MCF-7 cells) and α-tubulin (U937 cells) were determined after treatment with 50 μM of compound **2**. Selisistat (10 μM), suberoylanilide hydroxamic acid (SAHA, 5 μM), and AGK-2 (50 μM), a SIRT2-selective inhibitor,[28] were used as reference agents. In these assays, the level of Lys 373–382 acetylated p53 increased after treatment with **2**, correlating with p53 activation (Figure 1a). Similarly, the acetyl-α-tubulin level

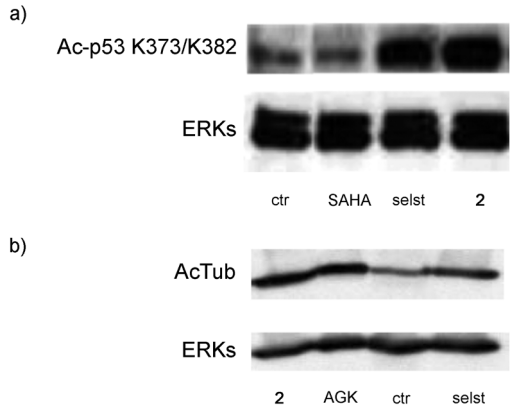


Figure 1. Western blot analyses performed with compound **2** (50 μM) to detect a) p53 or b) α-tubulin acetylation, in MCF-7 cells or in U937 cells, respectively. Selisistat (selst, 10 μM), SAHA (5 μM), and AGK-2 (50 μM) were used as reference agents.

showed an increase after treatment with either compound **2** or AGK-2 (Figure 1b). In this assay, the SIRT1-selective inhibitor selisistat also displayed a signal; while α-tubulin deacetylation is typically mediated by SIRT2, a recent report indicated that it can also be effected by SIRT1,[29] which could explain this observation.

Selected SIRT inhibitors **2**, **4**, **6**, **9**, and **11** were tested at 50 μM for 30 h in the human U937 leukemia cell line to detect their effects on cell-cycle progression and induction of apoptosis (Figure 2). As reference agents, carprofen and selisistat were also evaluated in the same assay at 50 μM. SAHA was included as a positive control for apoptosis induction. After treatment with either selisistat or compound **6**, cell-cycle arrest in the G1

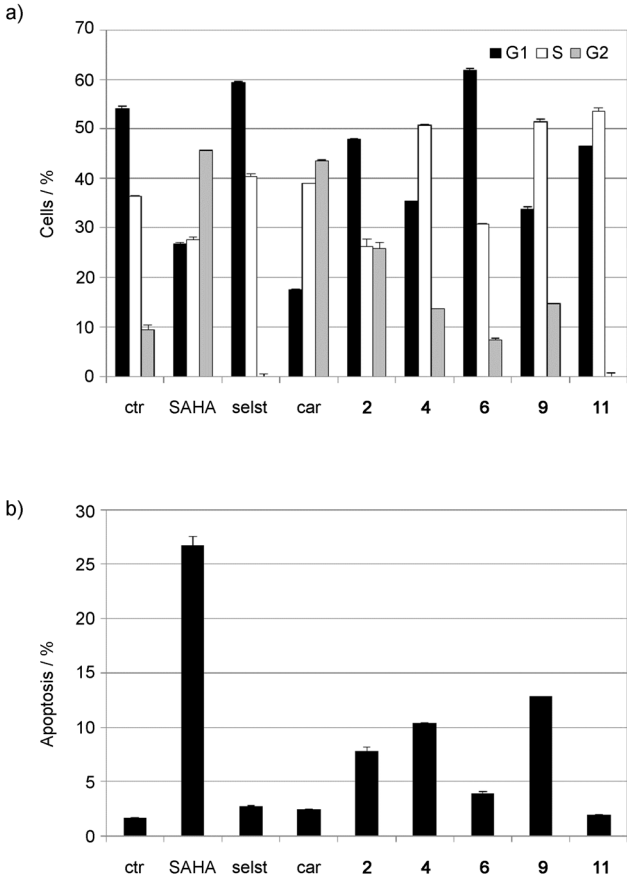


Figure 2. a) Cell cycle effect (proportion (%) of cells in a given phase of the cell cycle) and b) apoptosis induction (proportion (%) of apoptotic cells) of compounds **2**, **4**, **6**, **9**, and **11** in U937 cells (50 μM, 30 h). Carprofen (car, 50 μM), selisistat (selst, 50 μM) and SAHA (5 μM) were used as reference agents.

phase was observed, while treatment with compound **4**, **9**, or **11** led to an increase in the number of cells in the S phase (Figure 2a). With regards to apoptosis induction, treatment with compounds **2**, **4**, and **9** gave little increase in DNA fragmentation (pre-G1 peak), whereas selisistat and carprofen did not cause any detectable effect under the tested conditions (Figure 2b). In addition, granulocytic differentiation was evaluated by the increase in CD11c positive/propidium iodide (PI) negative cells caused by treatment with compounds **2**, **4**, **6**, **9**, and **11** at 50 μM in U937 cells over 24 hours. Known HDAC inhibitor entinostat (MS-275, SNDX-275) was used as the positive control. Under the test conditions, no induction of granulocytic differentiation was detected (see Supporting Information).

In conclusion, among the designed compounds, 2-(6-chloro-9*H*-carbazol-2-yl)propanamide **2** was the most efficient inhibitor of both SIRT1 and SIRT2, since further substitutions at the carboxamide function or at the carbazolyl nitrogen atom yield-

ed less potent or inactive compounds. Unlike selisistat and carprofen, compound **2**, and analogues **4** and **9** to a lesser extent, induced slight apoptosis in human leukemia U937 cells when tested at 50 μM for 30 hours. These results highlight the usefulness of the carbazole scaffold for the development of novel SIRT1/2 inhibitors. However, further studies are needed to improve the potency and selectivity of this novel class of compounds, as well as their ability to induce anticancer effects.

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Keywords: apoptosis · histone deacetylases · inhibitors · SIRT1/2 · sirtuins

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