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Brief Article

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Benzodeazaoxaflavins as Sirtuin Inhibitors with Antiproliferative Properties in Cancer Stem Cells

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Supporting Information

ABSTRACT: Inhibition of sirtuins has recently been proposed as a promising anticancer strategy. Some of the new benzodeazaoxaflavins (**2a**, **2b**, and **2d**) here reported as SIRT1/2 inhibitors were endowed with pro-apoptotic properties in human U937 leukemia cells and, most importantly, together with the prototype MC2141 (**1**) displayed antiproliferative effects in cancer stem cells from patients with colorectal carcinoma and glioblastoma multiforme, known to be highly tumorigenic, resistant to conventional cancer chemotherapy, and responsible, at least in part, for cancer relapse or recurrence.

INTRODUCTION

Class III histone deacetylases (HDACs), also indicated as sirtuins (SIRTs), are a family of seven enzymes in humans (SIRT1−7) which have NAD^+ as cosubstrate for their catalytic activity, show no homology with other classes of HDACs, and are sensitive to specific modulators.[1] Sirtuins catalyze the removal of the acetyl group from the lysine residues of their substrates, coupling this event with the hydrolysis of NAD^+ to generate nicotinamide, deacetylated protein, and *O*-acetyl-ADP-ribose.[1] Mono-ADP-ribosyl transferase (mART) activity has been found to be the only enzymatic activity of SIRT4, whereas SIRT1−3 and SIRT6 display both deacetylase (strong) and mART (weak) activities.[1,2] In addition to histones, SIRTs can deacetylate and/or ADP-ribosylate with different substrate specificities many other important proteins, such as transcription factors, enzymes, nuclear receptors, and other regulatory and structural proteins, so modulating their activity.[1−3] Very recently, SIRT5 has been found to be able to catalyze lysine demalonylation and lysine desuccinylation both in vitro and in vivo.[4]

These data can explain the multiple biological functions of sirtuins that range from repression of gene expression to regulation of circadian rhythm, DNA repair, cellular differentiation, and/or apoptotic processes, from control of energetic metabolism to aging.[1−3] Moreover, because these enzymes in humans regulate cell survival under stress conditions as well as neuro/cardio protection, stimulate insulin secretion and lipolysis, and inhibit adipogenesis, sirtuin activators have been proposed for the treatment of some age-related diseases and specific metabolic disorders.[1−3] SIRTs (SIRT1/3/7) have been found to be upregulated in many tumor types (such as leukemia, lung, and prostate cancer) and are able to inactivate at transcriptional and posttranslational level some tumor suppressor proteins such as p53 and p73 (SIRT1). In addition, sirtuins have been shown to activate the oncoprotein BCL6 (SIRT1) to exert antiapoptotic and antidifferentiation activities (SIRT1) through deacetylation of specific transcription factors (E2F1, FOXO3a, etc.) and to regulate DNA repair (SIRT1/6), cell cycle progression, and chromosomal stability (SIRT1/2). Thus, sirtuin inhibitors (SIRTi) have been proposed as potential anticancer agents.[5] However, the role of SIRTs in tumor progression is controversial, as these enzymes are downregulated in some cancer types and have potential tumor suppressor effects (SIRT1−3).[2,6] Because heterogeneous SIRT levels have been observed in subsets of tumors, it is conceivable that SIRTs display tumor suppressor or oncogenic functions depending on genetics and stage of the tumor and on the varying cellular environments and signals.[2,6] To date, only 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,[7] cambinol induced apoptosis in BCL6-expressing Burkitt lymphoma cells and mouse xenografts,[8] salermide, recently

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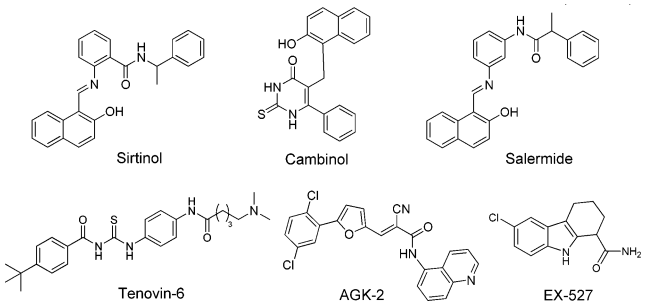
reported by us, showed tumor-specific apoptosis in a wide range of human cancer cell lines,[9] and tenovins decreased tumor growth in vivo as single agents at low micromolar concentrations (Chart 1).[10] Very recently, tenovin 6 in

Chart 1. Structures of Known SIRT Inhibitors (SIRTi)



combination with the BCR-ABL tyrosine kinase inhibitor imatinib was shown to effectively inhibit leukemia stem cells in chronic myelogenous leukemia (CML).[11] Differently, the SIRT1-selective inhibitor EX-527[12] (Chart 1) efficiently increased p53 acetylation in cells without effects on cell viability, or p53-controlled gene expression.[13]

Pursuing our efforts for identification of novel small molecule sirtuin modulators,[9,14−19] we recently identified the 10-phenyl-9*H*-benzo[5,6]chromeno[2,3-*d*]pyrimidine-9,11(10*H*)-dione MC2141 (**1**) as the prototype of a series of benzodeazaoxaflavins (BDF4s) as SIRTi endowed with significant inhibitory potency and high antiproliferative activity against Raji, DLD1, and HeLa cells.[17] Here we report the preparation and the biological evaluation of novel simplified analogues (**2a−g**, **3a−d**) of **1** aimed to improve its solubility and drug-like properties, to increase its pharmacodynamic properties, and to acquire structure−activity relationship (SAR) information (Figure 1).

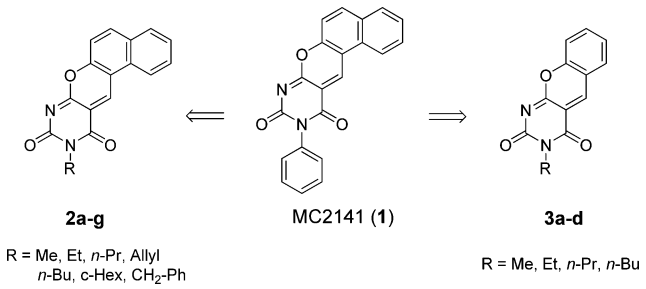


Figure 1. Novel MC2141 (**1**) analogues described in the present study.

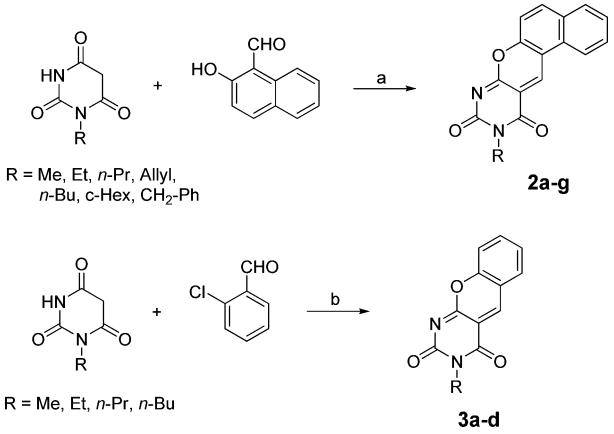
Because the previously reported *N*-unsubstituted analogue of **1** had shown similar inhibitory potency against SIRT1 as **1**, suggesting that the 10-phenyl substitution is not crucial for SIRT inhibition,[17] we introduced different (aryl)alkyl substituents at the *N*-10 position (**2a−g**), and we simplified the tetracyclic scaffold of the BDF4 prototype **1** by removal of the condensed benzene ring and by preparing a small series (**3a−d**) of different 3-substituted-2*H*-chromeno[2,3-*d*]pyrimidine-2,4(3*H*)-diones (also indicated as 5-deaza-10-oxaflavins) (Figure 1). All the synthesized compounds (**2a−g** and **3a−d**) were tested for their ability to inhibit the human recombinant SIRT1 and SIRT2 enzymes together with **1**, cambinol, AGK-2 (a SIRT2-selective inhibitor),[20] and EX-527 as reference drugs.

The effects of the novel compounds on cell cycle, apoptosis, and granulocytic differentiation in U937 cells have also been explored. Selected benzodeazaoxaflavins (**1**, **2a**, **2b**, and **2d**) have been finally tested on four cancer stem cell (CSC) lines for their effects on cell proliferation/survival. CSCs are a cancer cell subset highly tumorigenic, highly resistant to conventional cancer therapies, and supposed to play a crucial role in cancer relapse or recurrence.[21,22]

CHEMISTRY

The synthetic routes followed for the preparation of **2a−g** and **3a−d** are depicted in Scheme 1. The 10-substituted-9*H*-

Scheme 1



benzo[5,6]chromeno[2,3-*d*]pyrimidine-9,11(10*H*)-dione derivatives **2a−g** were prepared by condensation of the appropriate *N*-alkylbarbituric acids, synthesized by standard methods,[17] with 2-hydroxy-1-naphthaldehyde in dry ethanol at reflux temperature for 1 h. The 3-substituted-2*H*-chromeno[2,3-*d*]pyrimidine-2,4(3*H*)-dione compounds **3a−d** were obtained by condensation of the corresponding barbituric acids with *o*-chlorobenzaldehyde in ethanol dry in the presence of a catalytic amount of pyridine followed by the intramolecular dehydrohalogenation of the crude mixture of *E* and *Z* 5-(2′-chlorobenzylidene)barbituric acid intermediates by heating them in an oven at 240−260 °C without solvent for 0.5 h. The reference compounds **1** and cambinol have been prepared as previously reported by us.[17,18] EX-527 has been synthesized according to literature,[12] while AGK-2 was purchased from Sigma-Aldrich.

RESULTS AND DISCUSSION

The IC_{50} (inhibitory concentration 50, compound dose required to inhibit the enzyme activity of 50%) values for the novel compounds **2a−g** and **3a−d** against human recombinant (hr) SIRT1 and SIRT2 were determined using a fluorescent biochemical assay. As shown in Table 1, the majority of the newly synthesized compounds displayed SIRT1/2 inhibition with IC_{50} values in the low micromolar range. In particular, the introduction of a methyl or allyl substituent at the *N*-10 of the tetracyclic scaffold led to compounds (**2a,d**) more potent than the parent compound **1** against both the enzymes, while an ethyl or cyclohexyl group at the same position (**2b,f**) caused a decrease of the inhibitory activities of the derivatives, mainly against SIRT2. In the series of the tricyclic deazaoxaflavins **3a−d**, all the compounds retained inhibitory potency against SIRT1

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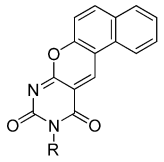
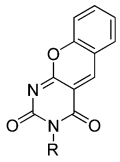
Table 1. Inhibitory Activity of 1−3 against Human Recombinant (hr) SIRT1 and SIRT2[a]



1, 2a-g



3a-d

compd	R	IC_{50}, μM hrSIRT1	IC_{50}, μM hrSIRT2
1	Ph	9.8 ± 0.3	12.3 ± 0.5
2a	Me	7.0 ± 0.2	11.2 ± 0.3
2b	Et	20.2 ± 0.8	30.0 ± 1.2
2c	*n*-Pr	9.4 ± 0.3	12.5 ± 0.5
2d	Allyl	6.6 ± 0.1	10.8 ± 0.3
2e	*n*-Bu	10.4 ± 0.4	16.9 ± 0.8
2f	c-Hex	21.1 ± 1.0	58.5 ± 2.9
2g	CH_2−Ph	12.0 ± 0.4	15.9 ± 0.6
3a	Me	10.5 ± 0.3	24.6 ± 1.0
3b	Et	11.8 ± 0.3	22.5 ± 0.9
3c	*n*-Pr	12.5 ± 0.5	29.9 ± 1.5
3d	*n*-Bu	14.1 ± 0.6	30.8 ± 1.5
cambinol		38% @ 150 μM	42.1 ± 2.1
AGK-2		38.0 ± 1.5	5.5 ± 0.2
EX-527		0.16 ± 0.008	48.5 ± 1.9

[a]Values are the means of two determinations.

comparable to the parent compound **1** with a slight decrease of activity along with the increase of the size of the alkyl substituent (from Me to *n*-Bu), and, with the only exception of **3b**, they were about 2-fold weaker than the corresponding tetracyclic counterparts against both SIRT1 and SIRT2. In the previous study, the lead **1** showed 8.4 and 191.2 μM inhibiting activities against SIRT1 and SIRT2, respectively.[17] Differently, under the present assay conditions, **1** resulted in activity against SIRT2 with IC_{50} values in the low μM range, too. Such SIRT2 inhibiting activity by **1** was also confirmed using a fluorescent acetylated lysine derivative, Z-MAL, that is widely employed for SIRT inhibition assays,[23] (see Supporting Information (SI), Table S3).

The novel SIRTi **2a−g** and **3a−d** were tested at 50 μM for 30 h in the human U937 leukemia cell line to detect their effects on cell cycle progression and apoptosis induction (Figure 2). Cambinol, EX-527, and **1** (all at 50 μM) were added as reference drugs. After the treatment, most of compounds **2** and **3** (in particular **2f** and **3a−d**) elicited cell-cycle arrest at the G_1 phase (Figure 2A). The FACS analysis for apoptosis indicated that all the novel derivatives **2** and **3** were able to induce programmed cell death more efficiently than EX-527 and cambinol (CMB), with compounds **2a**, **2b**, and **2d** being the most effective (18.8%, 23.5%, and 19.6% of apoptosis, respectively, Figure 2B) and even stronger than **1**.

In addition, granulocytic differentiation was evaluated for **2** and **3** (50 μM, 30 h) in U937 cells by the increase of % CD11c positive/propidium iodide (PI) negative cells. At the tested conditions, a significant induction of granulocytic differentiation was not detected (see SI).

Because of their pro-apoptotic properties, we selected **2a**, **2b**, and **2d** in addition to the lead **1** for evaluating their potential in the inhibition of cell viability of CSCs. We also added SIRT1-selective (EX-527) and SIRT2-selective (AGK-2) inhibitors to

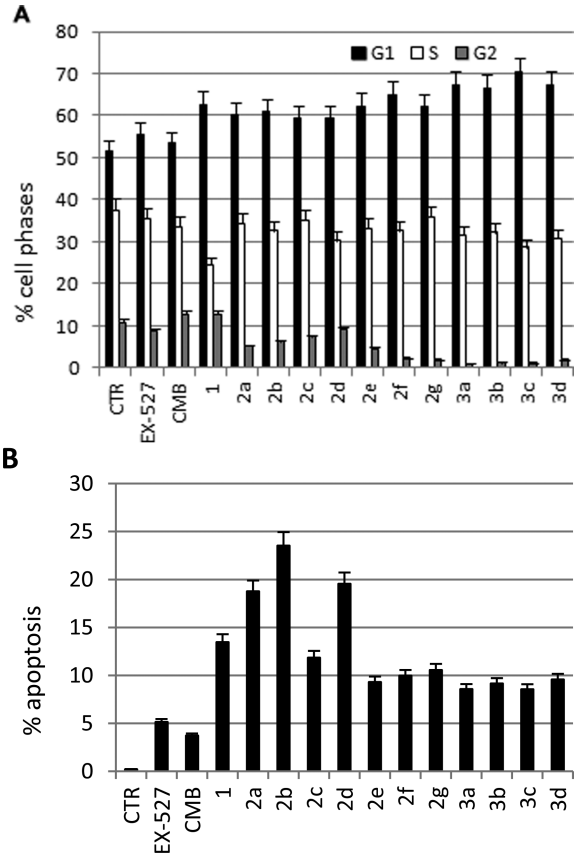


Figure 2. Cell cycle effect (A) and apoptosis induction (B) of **2a−g** and **3a−d** (50 μM, 30 h) in U937 cells. Values are the means of three experiments.

the assay in order to achieve information about the effect of isoform-selective SIRTi in CSCs. Two colorectal carcinoma (CRC) CSCs (CRO and 1.1 cells) and two glioblastoma multiforme (GBM) CSCs (30P and 30PT cells) isolated from patients were chosen for this assay. After 72 h of treatment, the CC_{50} (cytotoxic concentration able to kill 50% of the cells) values were determined and are reported in Table 2. The relative dose−response curves are reported in SI (Figure S2).

Against the tested CSCs, the SIRT1-selective inhibitor EX-527 failed to produce significant growth inhibition at 50 μM, whereas the SIRT2-selective inhibitor AGK-2 displayed good antiproliferative activity only against GBM CSCs. In contrast, compounds **1** and **2d** under the same conditions were highly

Table 2. Cell Viability Inhibition by 1, 2a, 2b, and 2d in CRC and GBM CSCs[a]

compd	CC_{50}, μM CRC CSCs CRO	CRC CSCs 1.1	GBM CSCs 30P	GBM CSCs 30PT
1	7.0 ± 0.6	5.5 ± 0.7	4.9 ± 0.2	3.9 ± 0.7
2a	75.4 ± 11.9	33.5 ± 10.7	34.2 ± 4.6	33.4 ± 2.8
2b	23.6 ± 2.6	14.9 ± 2.1	15 ± 1.7	15.6 ± 6.0
2d	9.6 ± 0.5	8.5 ± 0.4	6.6 ± 1.2	5.0 ± 3.8
EX-527	20%[b]	NI[b,c]	10%[b]	20%[b]
AGK-2	50%[b]	50%[b]	12.5 ± 0.5	9.6 ± 1.0

[a]Values are the means of three experiments. [b]Inhibition at 50 μM. [c]NI, no inhibition.

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efficient in reducing the viability of the four CSC lines tested, bearing CC_{50} values in the single-digit μM range. These effects seem to be quite sensitive to the substitution on the *N*-10 position of the tetracyclic system because the *N*-Me analogue **2a** showed a drop of antiproliferative activity of about 6−10-fold (depending on the specific cell line) in comparison with the lead **1**, and its *N*-Et counterpart **2b** exhibited a 3-fold lower potency in all the four CSC lines tested. Maybe a quite large, unsaturated substituent at *N*-10 seems to improve the cell penetration of the corresponding derivatives **1** and **2d**.

In conclusion, we have identified a series of low micromolar SIRT1/2 inhibitors endowed with pro-apoptotic properties in U937 human leukemia cells and, most importantly, with single-digit micromolar antiproliferative activity in CSCs from patients (coloncarcinoma and glioblastoma multiforme CSCs), which represent one of the most attractive targets of the modern anticancer therapy. Further studies are presently ongoing to improve the pharmacological profile of such novel compounds.

EXPERIMENTAL SECTION

Chemistry. Melting points were determined on a Buchi 530 melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 400 MHz on a Bruker AC 400 spectrometer; reporting chemical shifts in δ (ppm) units relative to the internal reference tetramethylsilane (Me_4Si). All compounds were routinely checked by TLC and ^{1}H NMR. TLC was performed on aluminum-backed silica gel plates (Merck DC, Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light. Yields of all reactions refer to the purified products. All chemicals were purchased from Aldrich Chimica, Milan (Italy) and were of the highest purity. Mass spectra were recorded on a API-TOF Mariner by Perspective Biosystem (Stratford, Texas, USA), samples were injected by an Harvard pump using a flow rate of 5−10 μL/min, infused in the Electrospray system. Elemental analyses were obtained by a PE 2400 (Perkin- Elmer) analyzer and have been used to determine purity of the described compounds, that is >95%. Analytical results are within ±0.40% of the theoretical values (see SI).

General Procedure for the Synthesis of 10-Substituted-9*H*-benzo[5,6]chromeno[2,3-*d*]pyrimidine-9,11(10*H*)-diones (2a−g). Example: 10-Propyl-9*H*-benzo[5,6]chromeno[2,3-*d*]-pyrimidine-9,11(10*H*)-dione (2c). A mixture of *N*-propylbarbituric acid (0.87 g, 5.1 mmol) and 2-hydroxy-1-naphthaldehyde (0.97 g, 5.6 mmol) in dry ethanol (22 mL) was heated at reflux temperature for 1 h. The hot suspension was then filtered off, and the resulting yellow solid purified by recrystallization from a mixture acetic acid/acetic anhydride (9:1); mp >260 °C; yield 85%. ^{1}H NMR (DMSO) δ 0.87 (t, 3H, CH_3), 1.58 (m, 2H, CH_2CH_3), 3.83 (t, 2H, $NCH_2CH_2CH_3$), 7.72−7.83 (m, 3H, C_{1-6}-*H* polycyclic system), 8.14 (m, 1H, C_{1-6}-*H* polycyclic system), 8.49 (m, 1H, C_{1-6}-*H* polycyclic system), 8.83 (m, 1H, C_{1-6}-*H* polycyclic system), 9.57 (s, 1H, C_{12}-*H* polycyclic system). ^{13}C NMR (DMSO) δ 10.8, 19.9, 46.16, 115.5, 116.3, 117.7, 122.3, 123.6, 126.8, 128.5, 128.7, 128.8, 130.1, 130.3, 150.5, 158.5, 160.0, 160.4 ppm. Anal. ($C_{18}H_{14}N_2O_3$) C, H, N. MS, *m*/*z*: 307 [M + H]. The chemical and physical data of the other compounds of general formula **2** are reported in SI.

General Procedure for the Synthesis of 3-Substituted-2*H*-chromeno[2,3-*d*]pyrimidine-2,4(3*H*)-diones (3a−d). Example: 3-Ethyl-2*H*-chromeno[2,3-*d*]pyrimidine-2,4(3*H*)-dione (3b). A mixture of *N*-ethylbarbituric acid (0.55 g, 3.5 mmol), *o*-chlorobenzaldehyde (0.54 g, 3.9 mmol), and a catalytic amount of pyridine (5 drops) in dry ethanol (14 mL) was heated at reflux temperature for 2 h. The mixture was then evaporated under reduced pressure, the residue triturated from diethyl ether, and, after filtration, the crude solid over the filter, without further purification, was heated in an oven at 260 °C for 0.5 h. After cooling at room temperature, the final solid was purified by recrystallization from a mixture acetic acid/acetic anhydride (9:1) to provide the desired product **3b** as a yellow solid; mp 236−238 °C (with decomposition); yield 67%. ^{1}H NMR (DMSO) δ 1.15 (t, 3H, CH_3), 3.88 (t, 2H, NCH_2CH_3), 7.57 (m, 1H, C_7-*H* polycyclic system), 7.73 (m, 1H, C_8-*H* polycyclic system), 7.92 (m, 1H, C_9-*H* polycyclic system), 8.12 (m, 1H, C_6-*H* polycyclic system), 9.01 (s, 1H, C_5-*H* polycyclic system). ^{13}C NMR (DMSO) δ 11.8, 38.5, 112.4, 114.2, 116.3, 121.2, 128.7, 128.9, 129.3, 154.3, 158.5, 160.0, 160.4 ppm. Anal. ($C_{13}H_{10}N_2O_3$) C, H, N. MS, *m*/*z*: 243 [M + H]. The chemical and physical data of the other compounds of general formula **3** are reported in SI.

SIRT1/2 Inhibition Assays. See SI.

Cell Lines. See SI.

Cell Cycle and Cell death Analysis on U937 cells. See SI.

Cell Viability Inhibition in Cancer Stem Cells (CSCs): Cell culture. CSC cultures were established and propagated under spherogenic conditions as described.[24,25] Cells were grown for 20−30 days in CSC serum-free medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) and maintained at a concentration of 50000−200000 cells/mL. Medium was replaced every three−five days with fresh medium complemented with 25% conditioned medium, depending on the growth rate and viability of each cell line. Cell viability assay. Spheroid cultures were enzymatically dissociated in Accumax Reagent (Sigma-Aldrich), cell density was determined, and single cell suspensions were dispensed into 96-well black microplates with clear, flat bottoms. Initial densities of 2000−3000 viable cells were chosen to be within the linear range of the assay at the chosen time point for all CSC lines. After plating, cells were incubated at 37 °C in 5% CO_2 for 24 h and then were treated with 1:3 serial dilutions of each compound (eight concentration points over a 0.023−50 μM concentration range) or with 0.1% DMSO as negative control. Three replicates for each experimental point were included. Cells were incubated at 37 °C in 5% CO_2 for 72 h, and then cell viability was determined using the CellTiter-Glo luminescent cell viability assay (Promega), essentially by following the manufacturer's recommendations. Compound cytotoxicity was evaluated in comparison to cells treated with DMSO. The CC_{50} values were determined by 4P logistic fitting of the experimental data with Kaleida Graph software based on the residual luminescence in the presence of increasing concentrations of the inhibitors.

ASSOCIATED CONTENT

Supporting Information

Chemical and physical data for compounds **2**−**3**. SIRT1/2 inhibition assays. Cell culture, cell cycle, and cell death induction analysis on U937 cells. Granulocytic differentiation investigation on U937 cells. Dose−response curves on CSCs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

HDAC, histone deacetylase; SIRT, sirtuin; FoxO, forkhead box class O; NAD^+, nicotinamide adenine dinucleotide; mART, mono-ADP-ribosyl transferase; NF-kB, nuclear factor-kappaB; BCL-6, B-cell lymphoma 6; BDF4s, benzodeazaoxaflavins; CMB, cambinol; FACS, fluorescence-activated cell sorting;

CSCs, cancer stem cells; CRC, colorectal carcinoma; GBM, glioblastoma multiforme; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor

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