SIRT1 Modulating Compounds from High-Throughput Screening as Anti-Inflammatory and Insulin-Sensitizing Agents

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The nicotinamide adenine dinucleotide (NAD$^+$)–dependent protein deacetylase SIRT1 has been linked to fatty acid metabolism via suppression of peroxysome proliferator-activated receptor gamma (PPAR-γ) and to inflammatory processes by deacetylating the transcription factor NF-κB. First, modulation of SIRT1 activity affects lipid accumulation in adipocytes, which has an impact on the etiology of a variety of human metabolic diseases such as obesity and insulin-resistant diabetes. Second, activation of SIRT1 suppresses inflammation via regulation of cytokine expression. Using high-throughput screening, the authors identified compounds with SIRT1 activating and inhibiting potential. The biological activity of these SIRT1-modulating compounds was confirmed in cell-based assays using mouse adipocytes, as well as human THP-1 monocytes. SIRT1 activators were found to be potent lipolytic agents, reducing the overall lipid content of fully differentiated NIH L1 adipocytes. In addition, the same compounds have anti-inflammatory properties, as became evident by the reduction of the proinflammatory cytokine tumor necrosis factor–alpha (TNF-α). In contrast, a SIRT1 inhibitory compound showed a stimulatory activity on the differentiation of adipocytes, a feature often linked to insulin sensitization. (*Journal of Biomolecular Screening* 2006:1-9)

Key words: SIRT1, small-molecule activators, HTS, adipocyte differentiation assay

INTRODUCTION

THE REGULATION OF COMPLEX NUCLEAR PROCESSES such as DNA replication, transcription, and repair takes places in a chromatin environment. The association of histone proteins with chromosomal DNA has been shown to exceed a mere packaging function but rather plays a fundamental role in gene regulation.[1] Histone-modifying proteins have emerged as a multiprotein family with different activities, and a variety of such posttranslational modifications have been unraveled and links of epigenetics to human disease established.[2] Among others, histone acetylation has been well studied, including the underlying enzymatic mechanisms leading to the characterization of the histone deacetylase (HDAC) family. This can be grouped into 3 classes due to a homologous histone deacetylase domain.[3] Class III of the HDAC family comprises the sirtuins or SIR-proteins (silent information regulator). However, these proteins have been considered unrelated to other HDAC classes due to their specialized functions in metabolic pathways.[4] SIR2 and its mammalian homologue SIRT1 were originally identified in yeast and are the best-characterized members of the sirtuin family.[5] SIRT1 is an evolutionarily conserved nicotinamide adenine dinucleotide (NAD)–dependent protein deacetylase, whereby this cofactor requirement is a distinct feature for all SIR proteins. NAD$^+$-dependence is absent in other protein deacetylases such as HDACs of classes I and II.

SIRT1 has been implicated in a variety of cellular processes such as metabolism, stress and DNA damage response, differentiation, and the control of multidrug resistance in cancer.[6-8] Extra copies of the gene encoding SIR2 have been correlated with longevity in the model organisms such as yeast, *Caenorhabditis elegans*, and *Drosophila melanogaster*. However, the effect of SIRT1 polymorphisms in humans could not be linked to longevity yet.[9,10] Besides targeting nuclear histones for deacetylation, SIRT1 has been implicated in removing acetyl-groups from transcription factors such as p53, the FOXO family, and NF-κB.[11-13] This regulation of the acetylation status has

several consequences on the cell cycle in response to activated p53 by promoting cellular survival under oxidative and/or DNA damaging stress. More recently, it has been shown that SIRT1 modulates NF-κB- dependent transcription by interacting with and deacetylating the active subunit of NF-κB (RelA/p65) in a site-specific manner, leading to its deactivation.[13] NF-κB plays a fundamental role as a proinflammatory transcription factor, thereby enhancing the expression of various cytokine genes, which are implicated in a variety of human diseases. NF-κB signaling plays an important function in amyloid beta-induced neuronal cell death, and SIRT1 has been shown to exert a protective function.[14] The list of the pleiotropic effects exerted by SIRT1 is growing, and published data revealed a role for SIRT1 in neuroprotection by preventing axon degeneration due to chemical or mechanical insults.[15] The latter finding has implications for the treatment of neurodegenerative diseases.

It has recently been shown that SIRT1 is a repressor of peroxysome proliferator-activated receptor gamma (PPARγ) expression, leading to fat mobilization in adipocytes.[16] This nuclear receptor is a key molecular mediator in adipogenesis and many underlying human disorders related to increased formation of adipose tissue.[17]

In summary, we have successfully identified classes of imidazoquinoxalines and pyrroloquinoxalines as SIRT1 activators, as well as tetrahydrocarbazole-based SIRT1 inhibitors.

This study characterizes these hits from high-throughput screening (HTS) to identify validated lead molecules based on cellular efficacy in established models of inflammation and obesity.

Although many compounds are used for these diseases, such as PPARγ agonists, some of these compounds cause undesirable side effects/toxicity or have a poor therapeutic response.[18] There is a need to develop new therapeutic agents with an improved therapeutic profile and reduced or no side effects/toxicity.

MATERIALS AND METHODS

Reagents and cell lines

3T3L1 mouse fibroblasts and THP-1 leukemia cells were purchased from ATCC (Manassas, VA) and cultured following the supplier's recommendations.

Cells were grown in suspension in complete RPMI 1640 culture medium (RPMI 1640; Invitrogen/Gibco, Rockville, MD), 2 mM glutamine (Invitrogen/Gibco), 10 mM HEPES, 1 mM sodium pyruvate (Invitrogen/Gibco), 100 μg/mL streptomycin, 100 U/mL penicillin, 0.05 mM 2-mercaptoethanol, and 10% fetal calf serum (FCS) at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. As the properties of the THP-1 cell were observed to change dramatically after prolonged periods in culture, only cells between passages 17 to 25 were used.

Viability of cells treated with the compounds described was routinely determined by a standard Trypan blue exclusion test.

Identification of SIRT1 activators and inhibitors

In an effort to discover modulators of the enzyme SIRT1, we conducted an HTS of 147,000 compounds against recombinant SIRT1 using a commercially available fluorescent assay. The screening library was acquired from more than 20 commercial vendors. Each vendor library was evaluated for its drug likeness. Compounds, which contained known toxicophores and reactive groups, were removed, and the remaining compounds were clustered and a diverse subset selected for purchase. All assay materials were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA), including recombinant SIRT1 enzyme (12.4 U/μL, SE-239), Fluor de Lys™ SIRT1-specific substrate (5 mM, KI-177), and Fluor de Lys™ Developer II Concentrate (5×, KI-176). We followed the published assay protocol.[19] Each reaction contained the assay buffer (25 mM Tris [pH 7.5], 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/mL bovine serum albumin [BSA]), 10 μM of the test compounds (for positive control, DMSO at a final concentration of 5% was added), 0.25 U SIRT1 enzyme (for negative control, neither enzyme nor compounds were added), and 100 μM Fluor de Lys™-SIRT1-specific substrate, and it was incubated at room temperature for 2 h. Fluor de Lys™ Developer II was added according to BIOMOL's recommendations, and the reaction was incubated for 45 min at 37 °C. Briefly, deacetylation of the substrate led to the generation of a fluorophore, which was excited at 360 nm and the emitted light (460 nm) detected on a fluorometric plate reader (Victor2 1420 multilabel HTS counter, Wallac, PerkinElmer, Boston). The signal window was calculated on the basis of positive (all reaction components except the test compound) and negative (all reaction components except the enzyme) controls placed in each plate. Mean value calculated from 16-well replicates of controls was applied in the calculation of activation capability. The sample activity was defined as the percentage of signal increase relative to signal window, according to the following formula: $100 \cdot (\text{Sample} - \text{Signal}_{\text{low}})/(\text{Signal}_{\text{high}} - \text{Signal}_{\text{low}})$. A value > 100% indicated a potential activation effect. To further confirm the activation effect, those compounds were retested with additional controls in the same fluorescent-based assay as previously mentioned. Each test was performed in duplicate plates, including 1 sample plate (with all reaction components) and 1 control plate (with assay buffer instead of the enzyme and all other components). The signal from sample plates was subtracted with that from control plates to eliminate compound autofluorescence. The subtracted signal was used for relative percentage of signal increase calculation. Compounds (at 10 μM) with a relative percentage over or equal to 150 were regarded as confirmed SIRT1 activators. The hit rate from the HTS campaign was 0.48%, and the IC_{50} values from the SIRT1 enzymatic assay revealed 3 hits to be < 1 μM, 34 hits between 1 and 5 μM, and 21 hits between 5 and 10 μM. The average Z' factor from all plates screened was 0.79 ± 0.02. The SIRT1

Table 1. Structures and Chemical Nomenclature for 3 Representative SIRT1 Activator Compounds from the Quinoxaline Series

COMPOUND	STRUCTURE	NAME
1		3-Benzenesulfonyl-1-(4-fluoro-phenyl)-1H-pyrrolo[2,3-b] quinoxalin-2-ylamine
2		2-Amino-1-(2-ethyl-phenyl)-1H-pyrrolo[2,3-b]quinoxaline-3-carboxylic acid (tetrahydro-furan-2-ylmethyl)-amine
3		2-Amino-1-(3-methoxy-propyl)-1H-pyrrolo[2,3-b]quinoxaline-3-carboxylic acid cyclopentylamide
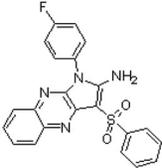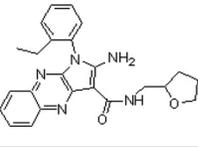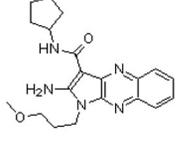
Table 2. Increase of Fluorescent Signal by SIRT1 activating compounds

Compound (at 10 μM)	% Signal Increase Relative to Signal Window
1	228 ± 71
2	216.5 ± 61
3	233 ± 13

Shown are average values (± standard deviation) from 2 independent experiments.

enzyme activation results for the compounds of **Table 1** are shown in **Table 2**. SIRT1 inhibitors were identified as compounds, which reduced the signal of the positive control to at least 50%. Those inhibitors were retested in dose-response experiments and IC_{50} values determined according to standard procedures.

Inhibition of tumor necrosis factor–alpha release in an inflammatory disorder model

The compounds identified as SIRT1 activators were tested in the following cell-based assay for their ability to inhibit tumor necrosis factor–alpha (TNF-α) release. This type of cell-based assay has been used as a model system to identify drugs for the treatment of inflammatory disorders.[20,21] THP-1 cells, a well-established line of human tumor cells, were treated first with representative compounds followed by addition of lipopolysaccharide (LPS) to induce the expression and release of TNF-α. The effect

of the test compounds on the expression of this proinflammatory cytokine TNF-α was thus studied.

THP-1 cells were plated at a density of 1.2×10^6 cells/mL in 0.4 mL (0.5×10^6 cells/treatment) in 24-well culture plates. The cells were preincubated with representative test compounds at 2 concentrations (20 or 60 μM) for 1 h under general tissue culture conditions (see above). The DMSO concentration used in all assays was 0.1%, and compound solubility was addressed by high-throughput solubility profiling and liquid chromatography/ mass spectrometry (LC/MS) analysis using the MassLynx Version 4.0 SP2 software (Waters Corporation, Milford, MA). LPS from *Escherichia coli* 055:B5 (Sigma) at final concentrations of 10 ng/mL was added to stimulate cytokine production in the cells.[22] Incubation was continued for another 5 h. Cells were harvested and centrifuged to collect the supernatant, which was used in the enzyme-linked immunosorbent assay (ELISA) assay.

ELISA was used to quantify TNF-α in the supernatant of THP-1 cells treated with the test compounds. TNF-α was measured in triplicate using double ligand immunoassays (Immunoassay Kit, BioSource International, Camarillo, CA). The detection limit of the ELISA was 15 pg/mL. All plates were read on a spectrophotometric microplate reader (SpectraMax Plus 384) and analyzed using a computer-assisted analysis program (ELISA End-point assay, Softmax Pro 4.0). Typically, standard curves generated with this ELISA were linear in the 100- to 1000-pg TNF/mL range.

Cell-based adipogenesis assay

The process of adipogenesis can be mimicked by a tissue culture model system comprising NIH3T3L1 mouse fibroblasts, a substrain of the Swiss 3T3 mouse cell line. These cells are commonly used to study the differentiation of the adipocyte phenotype induced by a combination cocktail consisting of dexamethason, isobutylmethylxanthine (IBMX), and insulin according to standard procedures.[23] The underlying biochemical pathways using this differentiation cocktail are well understood and involve a cascade of transcription factor inductions leading to overexpression of PPARγ. In the wake of cellular differentiation, cells change their appearance into the phenotype of mature adipocytes, which can be assessed microscopically. At the differentiated stage, cells produce lipid droplets, which are stained with Red Oil O. After washing procedures, pictures of the cells were taken, and the red dye was extracted with isopropanol following a spectrophotometric quantification.

The assay is a commonly used model for the discovery of PPARγ agonists and antagonists and is therefore suitable to identify compounds with antiobesity and antidiabetic properties. Representative compounds with SIRT1 activating properties were tested at a stage of fully differentiated adipocytes (i.e., reduction of lipids in this cellular system would reflect their ability to mobilize fat). Compounds were administered to the cells at increasing concentrations, starting from 5 μM. Fully

differentiated 3T3L1 cells were treated with an indicated amount of compound and stained with Red Oil O following standard procedures after 5 days. Incorporation of the red dye corresponded to high amounts of fatty acids and triglycerides produced by the cells.

Homology modeling and docking

An alignment between SIRT1 and the sequence of the X-ray structures of yeast Hst2 protein deacetylase and human SIRT2 (PDB entry 1Q1A, respectively 1J8F) was obtained by ClustalW and then manually edited.[24] The software programs used were purchased from Schrödinger (New York). The alignment was validated by comparing the predicted secondary structure of SIRT1 with that of 1Q1A and 1J8F. A SIRT1 homology model was built using Prime with standard settings. 1Q1A (40% homology to SIRT1) was chosen as a template as this is cocrystallized with a ligand (2′-O-acetyl ADP ribose). The homology model was side chain optimized using Prime with standard settings and energy minimized using the OPLS-2005 force field and generalized Born/solvent-accessible surface area (GB/SA) solvation model, as implemented in MacroModel.

The inhibitors were converted to 3D, and physiological relevant protonation states were generated using LigPrep. The protein structure was prepared, and grids were generated using the standard procedure, as recommended by the software provider. The compounds were docked using Glide with standard settings. Conformational energy calculation and analysis was performed using Monte Carlo multiple-minimum (MCMM) torsional sampling with manual setup, as well as the Merck molecular force field (MMFF) and GB/SA solvation model, as implemented in MacroModel.[25] The conformational energy was calculated as the difference between the global energy minima and the docked conformation minimized with flat-bottomed Cartesian constraints of 0.5 Å on all heavy atoms.

RESULTS

Identification of SIRT1 activators and inhibitors

An HTS campaign using a total of 147,000 compounds was carried out and primary hits selected. We defined a SIRT1 activator by a signal increase of 150% compared to the control plate, as described under Materials and Methods, whereas inhibitors were regarded as primary hits when their inhibitory activity was higher then 50%. Hits from the HTS were retested in hit confirmation assays to obtain average IC_{50} or EC_{50} values. **Tables 1** and **2** show the structure and properties of some of the SIRT1 activators identified.

Because the compound resveratrol has been identified by other groups as a potent agonist of SIRT1 activity in cell-based models and in vitro screens, we set up cell-based models, which helped to address potency and biological activity for selected compounds. SIRT1 has been shown to target PPARγ, leading to deacetylation in differentiating adipocytes and promoting lipolysis in white adipocytes.[16] We have tested the newly discovered SIRT1 activators in the well-known cell-based adipogenesis differentiation assay. In line with the published report on resveratrol effects in this system, we were able to demonstrate with some of our compounds a dose-dependent fat mobilization, as shown in **Figure 1** (representative data are shown for compound 3). Macroscopically, the intensity of Red Oil staining decreases in samples, which have received a higher dose of the compound (**Fig. 1A**). Quantification of the results obtained by dye extraction and absorbance reading, using a spectrophotometer, are described in detail in the Materials and Methods section and are shown in **Figure 1B**. The histological picture confirms this finding, whereby the size and number of intracellular cytosolic fat deposits are reduced in a dose-dependant manner (**Fig. 1C**). These results suggest that the compounds identified with SIRT1 activating properties have a significant effect on fat mobilization in differentiated adipocytes, and thus these compounds are anticipated to have antiobesity and/or antidiabetic properties.

Anti-inflammatory in vitro properties of SIRT1 activators

The transcription factor NFκB has been linked to inflammatory processes, and SIRT1 has been recently described to inhibit the transactivation potential of the RelA/p65 subunit.[13] Because biological activity of our SIRT1 activators could be confirmed in matured adipocytes, we established an ELISA-based assay to monitor TNF-α modulation comprising the human leukemia cell line THP-1, which is well known to produce and release TNF-α and other cytokines upon stimulation with LPS (as described in the Materials and Methods section). As summarized in **Table 3**, compounds with SIRT1 activating potential exert strong suppression of TNF-α release at concentrations between 20 and 60 μM. Resveratrol did not show a significant biological effect in this assay at comparable concentrations (**Fig. 2A**). Histograms of the dose-response experiments for the activator compounds 1 to 3 are shown in **Figure 2B**. Cell viability was controlled by Trypan blue exclusion for all experiments shown and did not reveal any cytotoxic effects for the compounds tested.

Inhibitors of SIRT1 stimulate lipogenesis in 3T3L1 cells

The HTS campaign led to the discovery of potent inhibitors of SIRT1 (a representative is shown in **Figure 3A**, compound 4), which were studied in the same biological assay as described above. The tetrahydrocarbazole compound 4 from the initial screen was found to be unstable and to decompose into an amide (5) as the major product. The chemical structure of compound 5 was confirmed by isolation from decomposed compound 4 and comparison of its analytical data (nuclear
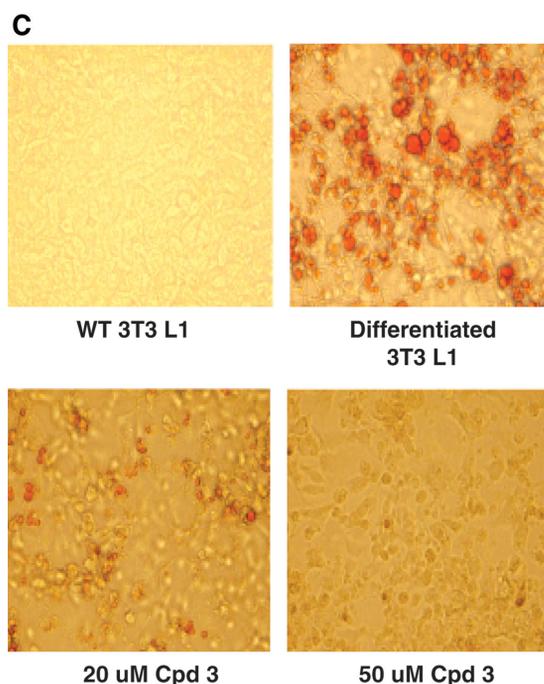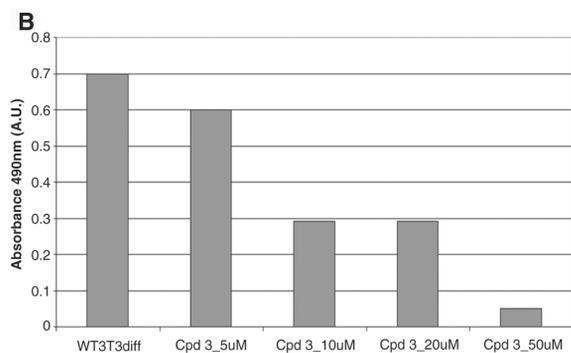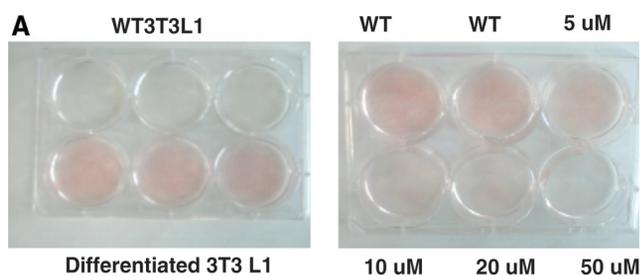
FIG. 1. Lipolytic activity of SIRT1 activator compound 3. (**A**) Dose-dependent decrease in Red Oil O–stained lipids upon treatment with increasing amounts (5 to 50 μM) of SIRT1 activator compound 3. Fully differentiated 3T3L1 fibroblasts were treated with increasing concentrations of the compound and stained as described in Materials and Methods. The experiment shown is a representative experiment out of 3 independent assays. (**B**) Triglyceride content of mouse adipocytes after treatment with SIRT1 activator compound 3 from the representative experiment shown in (**A**), quantified by Red Oil O extraction and absorbance reading. Shown are representative experimental data. (**C**) Histological pictures of treated samples and controls are shown in (**A**).

Table 3. ELISA-Based Quantification of TNF-α Suppression by SIRT1 Activator Compounds 1 to 3

Compound	Control	20 μM	60 μM
1	205.5 ± 20.8	116.4 ± 20.7	60.9 ± 3.3
2	248.3 ± 4.4	107.7 ± 1.4	50.6 ± 5.4
3	324.9 ± 39	103.7 ± 16.2	52.6 ± 12.7

The amount of TNF-α was measured as pg/mL after treatment of THP-1 cells at the indicated concentrations (shown as the average ± standard deviation). ELISA, enzyme-linked immunosorbent assay; TNF-α, tumor necrosis factor–alpha.
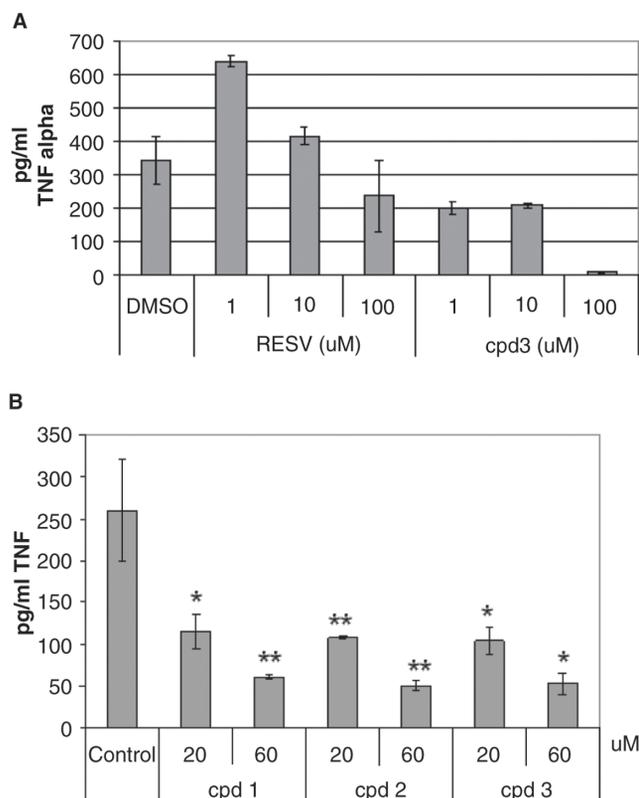

FIG. 2. Down-regulation of tumor necrosis factor–alpha (TNF-α) by compounds 1 to 3 in representative experiments using THP-1 cells. Results were quantified by a TNF-α-specific enzyme-linked immunosorbent assay (ELISA), as described in Materials and Methods. (**A**) SIRT1 activator compound 3 was compared with resveratrol (RESV) for the ability to decrease TNF-α expression triggered by lipopolysaccharide (LPS). The effective concentration was found to be between 10 and 100 μM. (**B**) Dose titration of compounds 1 to 3 in the THP-1 cell-based assay resulted in statistically significant reduction of TNF-α reduction compared to the control. The p-values from the unpaired t-test are < 0.005 for all compounds tested. **[*WHAT DO THE ASTERISKS (*, **) REPRESENT?*]**

magnetic resonance [NMR], LC/MS, and high-performance liquid chromatography/ultraviolet [HPLC/UV]) with those of authentic samples of compound 5. Compound 5 was found to be a potent SIRT1 inhibitor and was the major active component of

A

B

WT3T3 diff   + 0.1uM Rosiglitazone   + 1uM Rosiglitazone

+ 20 uM cpd 4   + 50 uM cpd 4

C
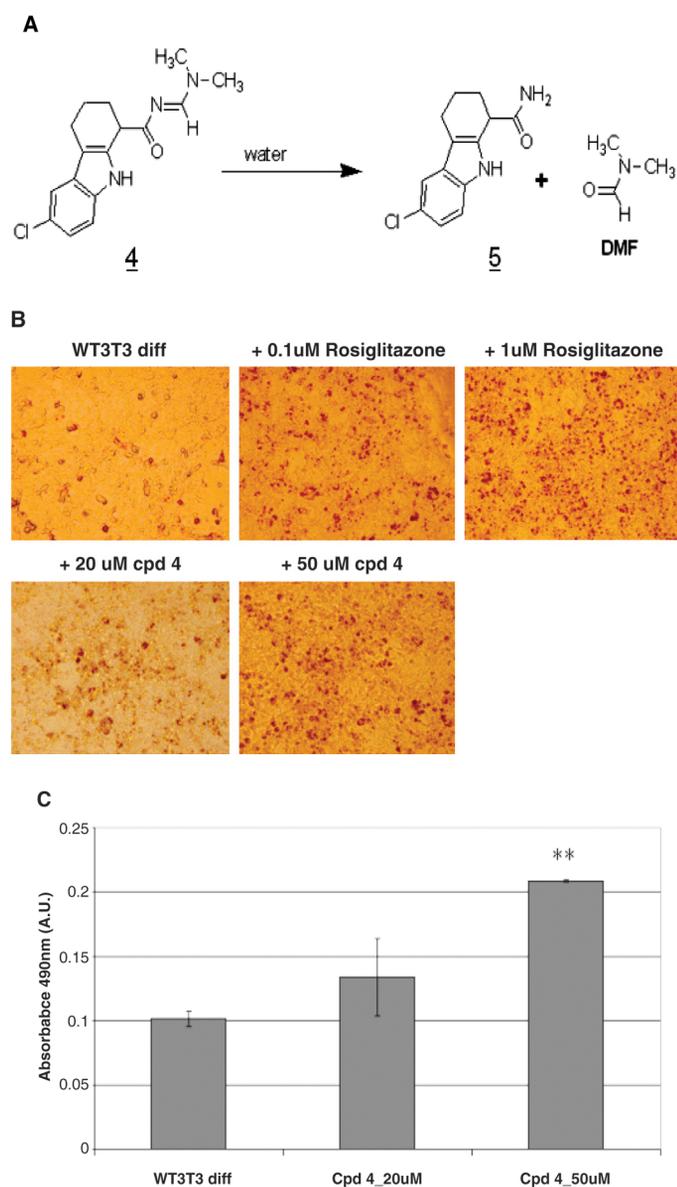
FIG. 3. Lipogenetic effect of SIRT1 inhibitor compound 4. (**A**) Structure of identified SIRT1 inhibitor: decomposition of screening compound 4 into an active amide (compound 5). (**B**) SIRT1 inhibitor compound 4 or the PPARγ agonist rosiglitazone was added to differentiating mouse 3T3L1 cells. (**C**) Matured adipocytes were stained as described and triglyceride content of mouse adipocytes after treatment with SIRT1 inhibitor compound 4 quantified. Values are given as mean ± SD ($n = 2$). The 2-tailed p-value for the 50-μM concentration of compound 4 compared to the control is < 0.0015 (**).

compound 4. During the period of preparing this manuscript, Napper et al.[19] also published that compound 5 was a potent SIRT1 inhibitor. Their results were in line with ours.

PPARγ activation is a result of direct interaction with agonists or, alternatively, based on the relief of a transcriptional block due to SIRT1 inhibition.[16] One obvious cellular change during the

course of differentiation from a fibroblast-like state to a fully matured fat cell is the increase in the number of lipid deposits. Known PPARγ agonists, such as the insulin sensitizer rosiglitazone, strongly enhance adipogenesis and differentiation, and we used this known compound as a reference for biological effects of SIRT1 inhibitors.[26] As shown in **Figure 3B**, rosiglitazone potently induces cellular differentiation at 100 nM, as becomes evident by an increase in the number of lipid deposits stained with Red Oil O. A 2-fold increase in the lipid content was observed for the SIRT1 inhibitor compound 4, although the concentration applied was 20 and 50 μM. Quantification was carried out by absorbance reading after Red Oil O extraction (**Fig. 3C**). This result confirms that modulation of SIRT1 activity leads to an opposite biological effect depending on the use of either specific activators or inhibitors.

SIRT1 inhibitor counteracts suppression of TNF-α

As enzymatic activity can be regulated by either agonists or antagonists, we carried out a proof-of-concept experiment for our activators and the potent SIRT1 inhibitor. By blocking SIRT1 function in THP-1 cells with the specific inhibitor (compound 4) prior to treatment with the SIRT1 activator (compound 5), we observed a lack of TNF-α suppression, when compared with the activator alone (**Fig. 4A,B**). The optimal time points for these compound treatments (pretreatment with SIRT1 inhibitor), followed by treatment with the SIRT1 activator and subsequent LPS-mediated TNF-α release, were determined empirically. This result demonstrates that the catalytic activity of SIRT1 can be regulated in opposing directions by either specific activators or inhibitors.

We propose the binding mode for this type of SIRT1 inhibitor compounds by homology modeling, as described under Materials and Methods. As shown in **Table 4**, all compounds except the inactive 11, 13, and 16 could be docked into the NAD⁺-binding site of the SIRT1 homology model with a binding mode that explained the observed structure-activity relation. Compound 4, which is unstable and is degraded into the amide compound 5 under assay conditions, did not dock either. All docked compounds had some affinity to SIRT1, and all docked in a conformation within 12 kJ/mol of the global energy minima. These are reasonable energies for bioactive conformations.[27] The predicted binding mode for active compound 5 is displayed in **Figure 4C**. R$_1$ docks into a small hydrophobic pocket formed by the backbone of Val412 and the side chains of Phe297, Ile347, Phe366, and Ile411. The indol nitrogen forms a hydrogen bond to Arg274. The ring system is positioned between the side chains of Phe273, His363, and Val445. The amide side chain falls into a hydrophilic pocket formed by the side chains of Arg274, Gln345, His363, Ser442, and the backbone nitrogen of Lys444. The amide forms hydrogen bonds to Gln345 and Lys444.

The less active compound 12, with a hydroxyl group at R$_1$, and compound 10, without any R$_1$ substituent, are lacking the
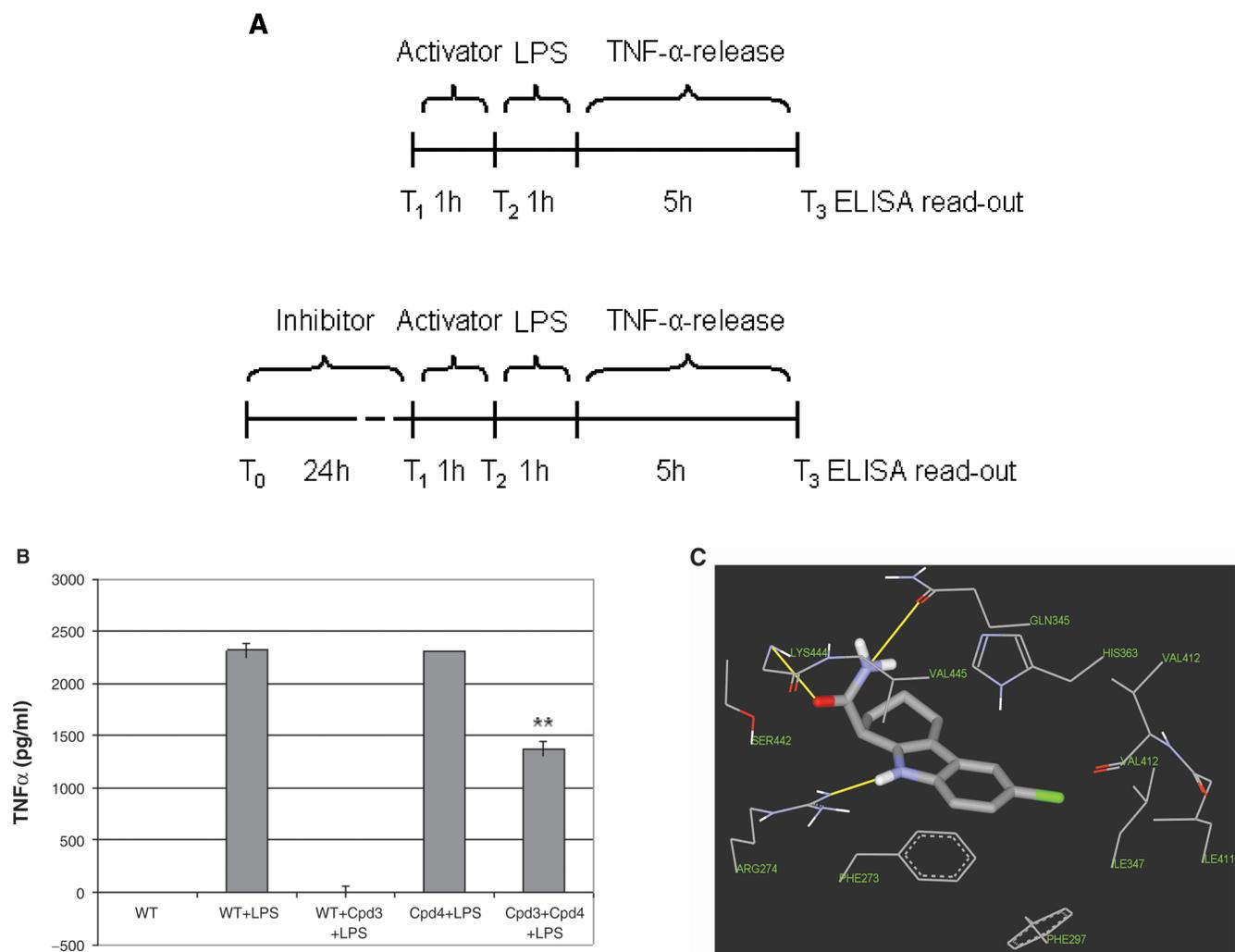
FIG. 4. SIRT1 inhibition overcomes activator-mediated tumor necrosis factor–alpha (TNF-α) suppression. (**A**) Experimental layout for the combination of SIRT1 inhibitor and activator in THP-1 cells. ELISA, enzyme-linked immunosorbent assay. (**B**) THP-1 cells were preincubated with SIRT1 inhibitor compound 4 for 24 h, followed by activating compound 3 and lipopolysaccharide (LPS). Readout was taken as for the experiments described in **Figure 2**. Data are given as mean ± SD (n = 3). The 2-tailed p-value for the combination of SIRT1 inhibitor and activator was determined to be < 0.0004. (**C**) Suggested binding mode of SIRT1 inhibitor compound 5 to the nicotinamide adenine dinucleotide (NAD$^+$)-pocket. Only the side chains of residues in the binding site are shown, except for Lys444, Val412, and Ile 411, for which backbone atoms are also shown. Hydrogen bonds between compound 5 and the protein are shown in yellow.

hydrophobic interactions with the side chains of Phe297, Ile347, Phe366, and Ile411. Compounds 6, 7, and 9 have a carboxylic acid at R$_2$ and form a hydrogen bond to the backbone nitrogen of Lys444 like the oxygen atom in the amide of 8. However, compounds 6, 7, and 9 lack the hydrogen bond to the side chain of Gln345.

DISCUSSION

We have identified potent SIRT1 modulators and characterized the altered SIRT1 activity in different biological systems. SIRT1 regulates complex cellular processes, and its increased activity in model organisms such as yeast, *C. elegans*, and *D.* *melanogaster* has been found to be associated with the extension of life span by slowing down aging processes.[9] In addition, the delay of aging processes upon SIRT1 activation in metazoans occurs by mimicking caloric restriction.[28] Due to its unique function as a NAD$^+$-dependent protein deacetylase, central to NAD$^+$-coupled metabolism and also cell cycle regulation via targeting of p53, modulation of SIRT1 activity may have therapeutic value for a variety of diseases such as cancer, inflammation, and metabolic disorders. Resveratrol, a natural product isolated from red wine, was the first SIRT1 activating compound reported and demonstrated to inhibit the transactivating potential of the RelA/p65 subunit of NF-κB via agonistic action on SIRT1 function.[13]

Table 4. Properties of SIRT1 Inhibitor Compounds 4 and 5
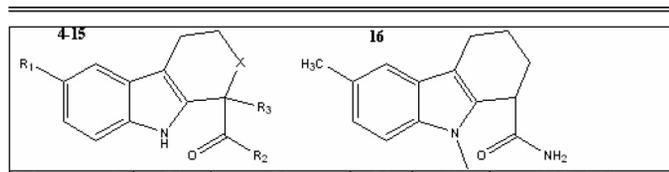
Compound	R1	R2	R3	X	IC50 (µM)
4	Cl	N=CHN(CH₃)₂	H	CH₂	0.149 ± 0.08
5	CH₃	NH₂	H	CH₂	(0.205)
6	CH₃	OH	H	CH₂	3.1 ± 0.8
7	OCH₃	OH	H	CH₂	2.8 ± 0.9
8	Cl	NH₂	H	CH₂	0.165 ± 0.07
9	H	OH	CH₃	NH	5.8 ± 1.6
10	H	NH₂	H	CH₂	(1.47)
11	Cl	OCH₂CH₃	H	CH₂	(>100)
12	OH	NH₂	H	CH₂	(15.0)
13	Cl	NHCH₂CH₃	H	CH₂	(>100)
14	Cl	NH₂	H	-	(0.409)
15	Cl	NH₂	H	(CH₂)₂	(0.124)
16					(>100)

Numbers in parentheses are average values taken from Napper et al.[19] (standard error < 30%, *n* = 3). Shown are the mean IC$_{50}$ values ± standard deviation from 2 independent experiments. The SIRT1 inhibitor compounds 4 and 5 are selective for recombinant SIRT1 and do not inhibit other members of the histone deacetylase (HDAC) family (data not shown).

TNF-α is one of the key cytokine mediators involved in the inflammatory response and is used as a marker for many inflammatory disorders.[29] The biological importance of TNF-α inhibition in the treatment of inflammatory disorders such as rheumatoid arthritis, Crohn's disease, and ulcerative colitis became apparent with the discovery and use of infliximab, a monoclonal antibody directed against TNF-α.[30] It has also been reported that NF-κB binding elements in the murine and human TNF-α gene promoters mediate TNF-α expression in response to LPS treatment.[31,32] Increasing the activity of SIR proteins, especially SIRT1, could be an effective new approach in the dysregulation of proinflammatory factors such as TNF-α.

Here we show that quinoxaline-based potent activators of SIRT1, discovered from a high-throughput screen, decrease TNF-α after LPS induction, as quantified by a sensitive ELISA assay. Resveratrol is a weak activator of SIRT1 function, thereby providing a precedented standard for comparative studies with these more potent activators. These compounds are at least 10-fold more potent in the in vitro suppression of TNF-α release compared to resveratrol. Another important function of SIRT1 in vivo is gene silencing, and 1 of the targets regulated by SIRT1 encodes the nuclear receptor PPARγ, which plays a central role in lipid metabolism and adipocyte differentiation. We therefore evaluated the quinoxaline compounds in a cell-based adipocyte differentiation system. PPARγ is the driving force of lipid accumulation in matured adipocytes, and resveratrol has been reported to enhance fat mobilization, an effect that could be confirmed with the more potent SIRT1 agonists in our assays.

The effect of SIRT1 activation is reversible by using a specific inhibitor, which we have identified in our HTS campaign (as shown in **Table 4**). This inhibitor compound is a tetrahydrocarbazole and is similar to a recently published indole showing SIRT1 inhibition.[19] SIRT1 is an enzyme whose activity can be modulated with either agonists or antagonists, and **Figure 4** illustrates a case whereby the effect of a SIRT1 antagonist counteracts the TNF-α-suppressing potential of an agonist. We studied the SIRT1 inhibitors in our cell-based lipogenesis assay as well and could show that down-regulation of PPARγ activity with a SIRT1 inhibitor leads to fat accumulation, a frequently observed effect seen in the treatment with insulin-sensitizing agents such as rosiglitazone. The intracellular accumulation of lipids is a hallmark for the insulin-sensitizing effect because it parallels an increased uptake of free fatty acids, which have been associated with insulin resistance, and fine regulation of the cellular lipid content may be dependent on the specific therapeutic indication addressed (e.g., obesity or type II diabetes).[33]

Many in vitro assays make use of artificial substrates, which contain a fluorophore or modified peptides, which carry only 1 residue such as an acetyl-group. In vivo, multiple modifications occur on correctly folded proteins at the same time, and it is conceivable that compounds such as resveratrol and the activators presented in this study have a specific mode of action. We have not further investigated this mechanism of SIRT1 activation, but it is tempting to speculate that resveratrol-like compounds, which increase SIRT1 activity, may in fact be allosteric effectors leading to stabilization of substrate binding. It is not known whether this allosteric mode of action will direct SIRT1 to low-affinity targets in vivo. This idea is supported by 2 recently published reports on the mechanism of SIRT1 activation by resveratrol.[34,35]

In summary, in an HTS campaign against human recombinant SIRT1 using a library comprising 147,000 compounds, we have successfully identified 2 classes of compounds, activators and inhibitors. We confirmed a set of quinoxalins with strong activating potential on recombinant SIRT1. Compounds, such as the SIRT1 activators 1 to 3 and the tetrahydrocarbazole-based SIRT1 inhibitor compound 4 presented here, have the ability to modulate the activity of SIR proteins such as SIRT1. They may therefore provide a new means of treating obesity and related metabolic disorders such as atherosclerosis, hypertension, type II or non-insulin-dependent diabetes mellitus, pancreatitis, hypercholesterolemia, hypertriglyceridaema, and hyperlipidemia, as well as a variety of inflammatory diseases. However, representatives of these compound families need to be further optimized in hit-to-lead programs comprising medicinal chemistry efforts to address important issues such as toxicity and specificity.

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