Emerging Therapeutic Potential of SIRT6 Modulators

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ABSTRACT: Sirtuin 6 (SIRT6) is an NAD⁺-dependent protein deacylase and mono-ADP-ribosyltransferase of the sirtuin family with a wide substrate specificity. In vitro and in vivo studies have indicated that SIRT6 overexpression or activation has beneficial effects for cellular processes such as DNA repair, metabolic regulation, and aging. On the other hand, SIRT6 has contrasting roles in cancer, acting either as a tumor suppressor or promoter in a context-specific manner. Given its central role in cellular homeostasis, SIRT6 has emerged as a promising target for the development of small-molecule activators and inhibitors possessing a therapeutic potential in diseases ranging from cancer to age-related disorders. Moreover, specific modulators allow the molecular details of SIRT6 activity to be scrutinized and further validate the enzyme as a pharmacological target. In this Perspective, we summarize the current knowledge about SIRT6 pharmacology and medicinal chemistry and describe the features of the activators and inhibitors identified so far.

INTRODUCTION

The sirtuin family is a class of enzymes that employs NAD⁺ as cofactor.1 Although initially classified as class III HDACs, sirtuins (SIRTs) are capable of catalyzing different reactions and possess a wide range of substrates far beyond histones.2 Among them, sirtuin 6 (SIRT6) is a pivotal chromatin homeostasis modulator that deacetylates both histone and nonhistone proteins, including DNA repair factors and glucose homeostasis regulators. In addition, SIRT6 promotes the deacylation of long-chain fatty-acid groups and catalyzes the mono-ADP-ribosylation of chromatin silencing DNA repair proteins,3 including self-mono-ADP-ribosylation.4 Through its enzymatic activity, SIRT6 facilitates the removal of acyl groups from the ε-amino group of lysines and transfers ADP-ribose moieties to lysine and arginine residues of protein substrates (Figure 1).

Given the requirement of NAD⁺ for their activity, SIRTs have been regarded as pivotal proteins connecting metabolism to cellular physiology.5 SIRT6, being a nuclear member of this family, tightly regulates DNA repair and genome maintenance and has a pivotal role in glucose and lipid metabolism. These activities are tightly related to the central roles that SIRT6 has in aging, stem cell differentiation, and tumorigenesis.

Loss-of-function studies performed in mouse models indicated the crucial roles that SIRT6 plays for organism wellbeing. Indeed, SIRT6-deficient mice displayed alteration of glycolysis and genomic instability, ultimately leading to premature aging and shortened lifespan.6–8 In addition, SIRT6 deletion was associated with increased tumor aggressiveness, and later studies in human cancers identified mutations impairing SIRT6 activity.9 Conversely, recent studies also described SIRT6 as a tumor promoter, hence highlighting the context-dependent role of this enzyme in cellular homeostasis.10,11

Homozygous mutations leading to SIRT6 loss of activity in humans caused fetal loss associated with muscle and brain developmental deficiencies.12 Similarly, cynomolgus monkeys bearing a SIRT6 knockout obtained through CRISPR-Cas9 suggested a primary role of SIRT6 for primates’ fetal development.13 Conversely, SIRT6 overexpression in male mice determined an increased lifespan, and another study indicated that SIRT6 levels increase in cultured cells, mice, and rats under conditions of caloric restriction, a dietary program that protects against many aging-related changes.14

SIRT6 has been initially described as an HDAC, having histone H3 as a substrate and catalyzing the deacetylation of lysines Lys9, Lys18, and Lys56.15–18 Histone deacetylation is associated with compaction of chromatin and consequent transcriptional repression as well as DNA-damage response. Nevertheless, recent reports indicated that SIRT6 deacetylase catalytic activity is 100 to 1000 times lower compared to the most active SIRTs.19 In addition, the deacylase efficiency of SIRT6 has been shown to be higher compared to deacetylation,
which can be in turn activated by small molecules, including free fatty acids (FFAs).\textsuperscript{20,21} For instance, \textit{in vitro} demyristoylation activity is roughly 300 times higher than deacetylation.\textsuperscript{22} Nonetheless, the majority of studies on SIRT6 indicate deacetylation as the main reaction responsible for its cellular functions, while deacylation has only been reported in the case of TNF-\textalpha{}\textsuperscript{22} and R-Ras\textsuperscript{223} so far. These features, along with the ability of SIRT6 to catalyze mono-ADP-ribosylation, depict a complicated picture of SIRT6 biological functions. Many details connecting the biochemical activity of SIRT6 and the observed phenotypes in both physiological and pathological conditions are still missing; hence, the main goals for future investigations consist of uncovering new SIRT6 substrates and elucidating its molecular interactors.

An important strategy for further elucidation of SIRT6 activity is played by chemical probes that through activation or inhibition of SIRT6 enzymatic activity may help to clarify the connection between SIRT6 function and the observed phenotypes. In addition, given the central role that SIRT6 plays in processes such as DNA repair, metabolism, aging, and tumorigenesis, small-molecule modulators could represent potential weapons for SIRT6-targeted treatment of diseases such as diabetes, obesity, cancer, and neurodegeneration.

**Figure 1.** (A) Deacetylation/deacylation reaction catalyzed by SIRT6. The acetyl/acyl group is transferred to an NAD\(^+\) acceptor, coupled with removal of nicotinamide. (B) Mono-ADP-ribosylation reaction. In this case, ADP-ribose is transferred onto the \(\varepsilon\)-amino group of lysine from an NAD\(^+\) donor. Nicotinamide and ADP-ribosyl protein are the products.

### SIRT6 STRUCTURE AND CATALYTIC MECHANISM

A key role in the investigation of SIRT6 function is played by the elucidation of its structural features. A decade has passed since the first structure of SIRT6 in complex with ADP-ribose has been solved,\textsuperscript{19} followed by the structure of SIRT6 bound to both ADP-ribose and myristoylated H3K9 peptide (Figure 2A).\textsuperscript{22} SIRT6 has two globular domains: a large Rossmann fold and a small zinc-binding region. The Rossmann fold consists of six \(\beta\)-sheets sandwiched between four \(\alpha\)-helices on one side and two \(\alpha\)-helices on the other side. This domain contains the NAD\(^+\) binding site as well as a hydrophobic pocket to accommodate the acyl chains of SIRT6 substrates. Di\textsuperscript{fferently from other SIRTs, SIRT6 has been reported to bind NAD\(^+\) in the absence of the acylated peptide.\textsuperscript{19} This feature is explained by the structural differences in the NAD\(^+\)-binding region. Indeed, SIRT6 lacks the cofactor-binding loop\textsuperscript{24–26} but presents a helix (\(\alpha3\)) that keeps its ordered structure even in the absence of the acylated peptide.\textsuperscript{19}

The hydrophobic channel is shaped by residues belonging to different loops engaging hydrophobic interactions with the fatty acyl chain, as shown by Lin and colleagues (Figure 2B).\textsuperscript{22} In the presence of a myristoylated peptide, the N-terminus of SIRT6, which covers part of the hydrophobic pocket, becomes structured. The structural ordering induced by the myristoylated peptide may facilitate the catalytic process and explain the higher catalytic efficiency of long-fatty-chain deacylation compared to...
deacetylation. It can also account for the increased deacetylation activity in the presence of FFA and small molecules. Nonetheless, there are no SIRT6 structures bound to an acetylated substrate; hence, this hypothesis is yet to be proven.

Finally, the zinc-binding motif is only structural and does not participate in the catalysis; this feature is shared by all SIRTs and differentiates them from class I, II, and IV HDACs possessing in the active site a zinc ion essential for catalysis. Notably, the in vitro deacetylase activity of SIRT6 is much lower than that of other SIRTs, probably because of SIRT6 peculiar structural features. Nevertheless, several cell-based assays suggested that the deacetylation is the most prominent activity of SIRT6, and H3K9 was indicated as the SIRT6 main substrate. This is explained by the fact that SIRT6 preferably associates with histones when they are packaged in nucleosomes. Conversely, SIRT1 exhibits higher deacetylation activity toward unpacked histones. Thus, interaction with packaged histones may trigger a transition toward an active SIRT6 conformation. Hence, SIRT6 activity depends on histone packaging, thereby being lower when tested in vitro using free histones. It is therefore possible that in the case of other substrates SIRT6 deacetylase activity is affected by the presence of interactors contributing to the formation of multiprotein complexes.

As mentioned above, SIRT6 deacylase activity has been reported to be higher than deacetylation. However, a functional role for SIRT6-mediated deacylation has only been described in the case of TNF-α and R-Ras2. Importantly, histone deacylation has only been indicated in preliminary in vitro studies. In the same study in which TNF-α deacylation was described for the first time, Jiang et al. also showed that SIRT6 can catalyze the removal of octanoyl, myristoyl, and palmitoyl groups from H3K9 and of myristoyl from H2BK12 using synthetic histone peptides as substrates. A subsequent analysis was performed using a chemical biology approach, in which the SIRT6-acylated substrate was the octenoylated H3 incorporated in the nucleosome. The terminal olefin selectively could react with a tetrazine probe allowing nucleosome labeling. This study suggested that SIRT6 catalyzes the efficient deacylation of H3K9, H3K18, and H3K27 while having low activity toward H3K4 and H3K23. Nonetheless, the precise physiological role of histones’ acylation/deacylation equilibria need further elucidation.

The SIRT6 residue Gly60 is pivotal for deacetylation; indeed, the G60A mutant has its deacetylase activity abolished while retaining deacylase activity. Mechanistically, Gly60 is crucial for NAD+ binding, and fatty-acylated substrates, but not acetylated ones, are able to reverse the conformational change induced by its mutation. This is in line with the above-mentioned activation of SIRT6-mediated deacetylation in the presence of FFA.

Beyond deacetylation and deacylation, SIRT6 also catalyzes mono-ADP-ribosylation. This was initially demonstrated using

Figure 2. (A) Structure of SIRT6 in complex with H3K9-Myr (green) and ADP-ribose (yellow) bound (PDB ID: 3ZG6). (B) Focus on the hydrophobic pocket in the Rossman fold accommodating the myristoyl chain. (C) Catalytic mechanism of SIRT6-mediated deacylation.
mouse SIRT6 (mSIRT6), which was shown to self-mono-ADP-ribosylate, and suggested that SIRT6 may self-modulate its activity through this post-translational modification (PTM). Further studies indicated that SIRT6 mono-ADP-ribosylates different factors, including the poly(ADP-ribose) polymerase 1 (PARP1), the transcription factor KAP1, the BAF chromatin remodeling complex subunit BAF170, and the histone lysine demethylase KDM2A.

Given its peculiar structure, SIRT6 can bind NAD$^+$ before the acylated protein, and following binding of both substrates, a slow conformational change allows the formation of the alkylimidate intermediate (Figure 2C, step I). The rate of this step is enhanced by FFA and small molecules and has been shown to be faster during demyristoylation. This reaction consists of a nucleophilic attack of the acyl carbonyl on C1' of nicotinamide-bound ribose and consequent formation of a C1'-O-alkylimidate intermediate, along with release of nicotinamide. Subsequently, His133 acts as a general base on ribose C3' thereby triggering an intramolecular nucleophilic attack of the C2' hydroxyl toward the C1'-O-alkylimidate, thus yielding the C1'-C2' cyclic intermediate (Figure 2C, step II). A conserved water molecule then catalyzes the hydrolysis of the cyclic intermediate, affording the tetrahedral intermediate (Figure 2C, step III). The imino group then attacks His133, which is now positively charged, thus gaining a proton and resulting in the cleavage of the C=N bond. This leads to the final products O-acetyl-ADP-ribose and deacylated lysine (Figure 2C, step IV), which are then released from the enzyme (Figure 2C, step V).

### BIOLOGICAL FUNCTIONS AND DISEASE RELEVANCE OF SIRT6

**Genome Maintenance.** Initial observations on SIRT6-knockout mice revealed hypersensitivity to DNA-damaging agents and genomic instability, indicating aberrant functioning of DNA double-strand break (DSB) repair and base excision repair (BER) mechanisms. Following these early studies, a growing body of reports indicated that SIRT6 associates with damaged chromatin sites and coordinates the recruitment of different factors to initiate DNA-damage repair (DDR). In particular, Onn and colleagues suggested that a SIRT6 dimer is able to directly bind to open-ended DSBs, whereby each monomer interacts with one DNA strand.

SIRT6-mediated recruitment of repair factors is triggered by the deacetylation of nucleosomes. For instance, H3K6 acetylation facilitates the recruitment of the ISWI-chromatin remodeler SNF2H, which increases chromatin accessibility, thereby promoting the binding to damaged DNA of repair factors such as BRCA1, RPA, and 53BP1. Remarkably, a recent study indicated the crucial role of the SIRT6-SNF2H dimer at the neurological level. Indeed, animals lacking SIRT6 in the brain showed AD symptoms, including increased levels of hyperphosphorylated Tau protein.

Recent studies indicated that SIRT6 is phosphorylated on Ser10 by c-Jun N-terminal kinase (JNK) under oxidative stress conditions. This PTM enables the binding of SIRT6 to DSBs and subsequent recruitment of PARP1, which mediates nonhomologous end-joining (NHEJ) and homology-directed repair (HDR). PARP1 is also mono-ADP-ribosylated by SIRT6 on Lys521, a modification that is required for PARP1 activity in BER. In addition, mono-ADP ribosylation of the histone lysine demethylase KDM2A has been shown to augment H3K36me2 level at DNA-damage sites, thereby promoting H3K9 trimethylation and consequent recruitment of NHEJ factors to DSBs (Figure 3).

As anticipated above, the role of SIRT6 in DNA repair has implications in pathology and therapy, particularly in neurodegeneration as the frequency and precision of repair mechanisms declines with age. Accordingly, SIRT6 levels have been shown to decrease with cellular senescence and its overexpression is able to stimulate HDR through the PARP1 pathway.

SIRT6 mediates DNA repair also through BER as indicated by reports showing that overexpression of SIRT6 increases 2-fold the efficiency of this DNA repair mechanism. Moreover, under oxidative DNA damage, SIRT6 interacts with and stimulates MYH DNA glycosylase and the endonuclease APE1, two enzymes involved in BER. The process is aided by the checkpoint clamp Rad9-Rad1-Hus1 (9-1-1), which forms a multiprotein complex with MYH, APE1, and SIRT6 that is pivotal for whole genome and telomere stability in mammalian cells (Figure 3).

SIRT6 is also responsible for telomeric preservation in mammalian cells through deacetylation of H3K9 and H3K56 in telomeric regions. SIRT6-mediated H3K9 deacetylation determines chromatin conformational changes that allow the binding of the Werner syndrome ATP-dependent helicase (WRN), the DNA-processing factor that is mutated in the Werner syndrome, a premature aging disorder. Furthermore, SIRT6 interacts with telomere repeat binding factor 2 (TRF2), a pivotal regulator of telomere homeostasis and DNA-damage response, and their interaction is increased during DNA-damage events, in a PARP1-dependent manner. SIRT6 catalyzes TRF2 deacetylation, triggering its ubiquitination finally leading to its proteolysis. In line with this, the levels of the two proteins were negatively correlated in a cohort of colorectal cancer (CRC) patients. These results indicate a regulation mechanism of TRF2...
levels in response to DNA damage and oncogenesis, whereby SIRT6-induced degradation of TRF2 impairs DNA-damage repair leading to cancer cell death.44

Apart from its roles in DNA repair and telomeres, SIRT6 is mainly responsible for transcriptional silencing. SIRT6-mediated H3K9 and H3K56 deacetylation contributes to the repression of proteins involved in lipid metabolism, inflammation (NF-κB-dependent proteins), as well as c-Myc targets, ribosomal proteins, and early developmental genes.8,45−47 Furthermore, SIRT6 promotes the silencing of long interspersed element-1 (LINE-1) retrotransposable elements (RTEs), a class of retrotransposons linked to mutagenesis and genomic instability.48 SIRT6 facilitates heterochromatin packaging of these RTEs, hence suppressing transposition. Notably, recent findings indicate that this function is directed by SIRT6-mediated mono-ADP-ribosylation of the transcriptional corepressor KAP1 (Figure 3).32

Moreover, SIRT6 is responsible for pericentric chromatin silencing through H3K18 deacetylation, and this function is mediated by KAP1, although in a different manner compared to LINE-1 RTEs. Evidence indicates that H3K18 deacetylation is necessary for KAP1 retention at pericentric satellite repeats and consequent transcriptional repression. Conversely, SIRT6 knockout and consequent H3K18 hyperacetylation causes KAP1 detachment and transcriptional derepression.18 SIRT6-deficient cells display accumulation of pathological pericentric transcripts causing genomic instability, mitotic errors, and cellular senescence, defects associated with aging and tumorigenesis.

As previously mentioned, SIRT6 has also been indicated to catalyze the ADP-ribosylation of the BAF chromatin remodeling complex subunit BAF170 at Lys312. This modification enhances the transcription upon oxidative stress of a subset of the nuclear factor erythroid 2-related factor (NRF2) responsive genes such as HO-1.53

**Stem Cell Differentiation.** Embryonic stem cell (ESC) pluripotency maintenance is guaranteed by the expression of Oct4, Sox2, and Nanog genes, which are lost upon differentiation.49,50 Recent studies indicated that SIRT6 mediates ESC differentiation through H3K9 and H3K56 deacetylation, which determines the repression of ten-eleven translocation methylcytosine dioxygenase 1 and 2 (TET1 and TET2). These enzymes convert 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC) and regulate cell lineage choice during ESC differentiation (Figure 3).51

SIRT6 has also a role in mesenchymal stem cell (MSC) and hematopoietic stem cell (HSC) homeostasis through H3K56 deacetylation. Through this activity, SIRT6 seems to coactivate transcription of NRF2 target genes and protect MSCs from oxidative stress.52 In addition, SIRT6-mediated H3K56 deacetylation was shown to suppress the NF-κB signaling pathway, thereby promoting osteogenic differentiation and new bone formation and repair in rats.53 In case of HSCs, SIRT6 interacts with LEF1 and, through H3K56 deacylation, corepresses Wnt target genes, thus blocking aberrant HSC proliferation.54

In addition, SIRT6 expression is associated with higher reprogramming efficiency of induced pluripotent stem cells (iPSCs).55 Given the increasing evidence supporting iPSC-based therapies in the context of neurodegenerative diseases, SIRT6 activation may represent a useful strategy to increase the success rate of these treatments.

**Aging.** Given its roles in genomic maintenance and stem cell homeostasis, SIRT6 has an indirect influence on aging, a process tightly related to DNA damage, telomere maintenance, and differentiation. In addition, SIRT6 plays a direct role in senescence and aging-related conditions through its activity on specific substrates at both the cytoplasmic and nuclear level.

SIRT6 overexpression determined a 15% increase of male mice life expectancy along with reduction of insulin-like growth factor 1 (IGF-1) levels and extended maximal lifespan in both male and female C57Bl/6 mice.56−58

**Figure 3.** SIRT6 promotes the silencing of long interspersed element-1 (LINE-1) retrotransposable elements (RTEs), a class of retrotransposons linked to mutagenesis and genomic instability.48 SIRT6 facilitates heterochromatin packaging of these RTEs, hence suppressing transposition. Notably, recent findings indicate that this function is directed by SIRT6-mediated mono-ADP-ribosylation of the transcriptional corepressor KAP1 (Figure 3).32

**Figure 4.** Roles of SIRT6 in cancer. The figure indicates the main factors modulated by SIRT6 in the context of both tumor suppression and promotion. Different mechanisms are involved, including the regulation of DNA-damage response, glycolysis, apoptosis, cell migration, and inflammation.

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factor 1 (IGF1) signaling through increased levels of IGF-binding protein 1 (IGFBP1) and altered phosphorylation of proteins involved in IGF1 downstream signaling. Moreover, SIRT6 deacetylates the cyclin-dependent kinase inhibitor p27, a factor involved in cellular senescence, hence promoting its proteasome-dependent degradation. Similarly, the transcription factor NF-κB, which induces the expression of aging-related genes, is negatively regulated by SIRT6 through a double mechanism. Indeed, at the transcriptional level, SIRT6 deacetylates H3K9 at NF-κB promoters, thereby reducing the expression of its components, while at the protein level, SIRT6 catalyzed deacetylation of the NF-κB p65 subunit (RelA) at Lys310 results in NF-κB nuclear export (Figure 3). The tumor-suppressor role of SIRT6 was associated with the suppression of glycolytic genes crucial for the Warburg effect, a metabolic shift common in cancer cells where ATP is obtained mostly through glycolysis rather than mitochondrial oxidative phosphorylation, in order to generate immediate energy to support fast proliferation and related cellular processes. These genes, including the glucose transporter-1 (GLUT1), lactate dehydrogenase (LDH), phosphofructokinase-1 (PFK1), and pyruvate dehydrogenase kinase-1 (PDK1), are regulated by the hypoxia-inducible factor 1α (HIF-1α), which is corepressed by SIRT6. SIRT6 also deacetylates pyruvate kinase M2 (PKM2), a nuclear isoform that enhances aerobic glycolysis even under hypoxia conditions and promotes tumor growth. SIRT6-mediated deacetylation triggers PKM2 transport to the cytoplasm and repression of its functions. In addition, glycolytic genes are downregulated through direct deacetylation of H3K9 at their promoters.

In line with this, SIRT6 is selectively downregulated in CRC and pancreatic ductal adenocarcinoma (PDAC), which display increased expression of glycolysis-related genes. The following studies confirmed these findings and expanded the role of SIRT6 as a main regulator of glycolysis in prostate, bladder, and breast cancers. Interestingly, SIRT6 activity is antagonized by the Runt-related transcription factor 2 (RUNX2), which represses SIRT6 transcription in low-glucose conditions. In addition, E2 transcription factor 1 (E2F1) negatively regulates SIRT6 in response to hypoxia, hence facilitating the Warburg effect.

SIRT6-mediated H3K9 deacetylation has effects on multiple oncogenes beyond glycolytic genes. These include two proteins involved in apoptosis inhibition and consequently tumor progression: the caspase activation inhibitor survivin and the RNA-binding oncofetal protein Lin28b. Liver cancer mouse models also showed that survivin activity is impaired through the inhibition of NF-κB activation and consequent binding to a survivin promoter. NF-κB is also involved in the activation of other antiapoptotic proteins (FLIP, c-IAP1/2, and XIAP) and its expression is antagonized by SIRT6 in nasopharyngeal carcinoma (NPC). Lin28b expression is downregulated through deacetylation of both H3K9 and H3K56. In PDAC, SIRT6 deficiency was associated with H3K9 and H3K56 hyperacetylation at the Lin28b promoter and poor patient prognosis; moreover, in a mouse model of pancreatic cancer, a SIRT6 deficit led to increased tumor aggressiveness and metastasis. Given the severity of PDAC and the important role played by SIRT6 in this subset of tumors, targeting this pathway through activation of SIRT6 may represent a successful approach for this type of malignancy.

Lin28b is also a target gene of the c-Myc oncogene. In PDAC, Lin28b promoter hyperacetylation is associated with c-Myc recruitment and consequent augmentation of cancer progression and metastasis. Notably, c-Myc activity is antagonized by SIRT6, which represses the transcription of c-Myc and its target genes and leads to cell cycle arrest and inhibition of tumor growth. In DLBCL cells, knockdown of SIRT6 leads to higher sensitivity to ERK2 and p90 RSK signaling increases resistance to DNA-damaging therapeutics. Upregulation of SIRT6 has also been observed in other blood cancers, including acute myeloid leukemia, chronic lymphocytic leukemia, and diffuse large B-cell lymphoma (DLBCL). In DLBCL cells, knockdown of SIRT6 leads to higher sensitivity to chemotherapy, altered cell proliferation, augmented rates of apoptosis, and cell cycle arrest. These phenotypes were associated with inhibition of the PI3K/AKT/mTOR signaling pathway.

SIRT6 catalytic activity determines an increase of the intracellular ADP-ribose concentration, which activates the Ca²⁺ channel TRPM2. Increased Ca²⁺ concentration finally leads to the activation of the Ca²⁺-dependent nuclear factor of activated T cells (NFAT), which upregulates the expression of TNF-α and IL-8, two proangiogenic and proinflammatory cytokines that promote tumor growth and metastasis.
A recent study indicated that SIRT6 is overexpressed in NSCLC cells, and its silencing determined activation of the p53/p21 pathway and consequent inhibition of cell proliferation associated with cell cycle arrest and apoptosis. Conversely, an earlier study indicated that SIRT6 suppresses NSCLC proliferation through inhibition of Twist1 expression, a factor that facilitates EMT and metastasis.

These examples indicate the complicated role played by SIRT6 in tumorigenesis, suggesting a context dependency. If we take into account the involvement of SIRT6 in DNA-damage

| Compound | Structure | Effect on SIRT6 activity | Cellular and in vivo effects | Ref |
|----------|-----------|--------------------------|------------------------------|-----|
| 1a Myristic acid | | EC50 = 246 μM × 10.8 max activation (deacetylation) | - | 20 |
| 2b OEA | | EC50 = 3.1 μM × 2.1 max activation (deacetylation) | - | 102 |
| 3e Cyanidin | | EC50 = 460 μM × 55 max activation (deacetylation) | In Caco-2 cells: dose-dependent SIRT6 upregulation; increased expression of FoxO3a. Decreased expression of Twist1 and GLUT1. | 103 |
| 5 UBCS039 | | EC50 = 38 μM × 3.5 max activation (deacetylation) | SIRT6 activation in NSCLC, colon and epithelial cervix carcinoma, and fibrosarcoma. Decrease of H3K9 and H3K56 acetylation and autophagy-related cell death. | 124, 125 |
| 7a MDL-800 | | EC50 = 10.3 μM × 22 max activation (deacetylation) | Dose-dependent decrease of H3K9Ac and H3K56Ac in HCC and NSCLC causing cell cycle arrest. HCC tumor growth suppressed also in mouse xenograft models. | 128, 129 |
| 7c MDL-811 | | EC50 = 5.7 μM (deacetylation) | Dose-dependent reduction of H3K9Ac, H3K18Ac, and H3K56Ac levels in different CRC cell lines and antiproliferative effects associated with marked G0/G1 cell cycle arrest. CRC growth suppressed also in patient-derived organoids and antitumor efficacy in cell line-derived and patient-derived xenografts. | 132 |
| 8b CL5D | | EC50 = 15.5 μM (deacetylation) × 50 increase in $kcat/Km$ $K_i$ = 13.4 μM (demyristoylation) | Time-dependent SIRT6 deacetylase activity enhancement in full length histones extracted from HEK293T cells | 3 |
| 10b | | EC50 = 5.35 μM (deacetylation) EC90 = 8.91 μM (demyristoylation) | Rise in SIRT6 melting temperature at 25 μM (CETSA). Suppression of PDAC cells proliferation cell cycle arrest in G2. Antitumor activity in a human pancreatic tumor xenograft mouse model associated with decrease of H3K9 acetylation levels. | 138 |
repair, depending on the stage of cancer progression, this pathway may have tumor-promoting or tumor-suppressing effects. Moreover, high levels of SIRT6 associated with tumors may also represent a compensating response rather than a causality. Therefore, it is vital to distinguish the potential of SIRT6 as a therapeutic target or as a biomarker in each type of tumor.

**Inflammation and Immunity.** In the context of immune regulation, SIRT6 has been shown to upregulate proinflammatory cytokines, as explained above in the case of TNF-α and IL-

### Table 2. Most Relevant SIRT6 Inhibitors

| Compound      | Structure | Effect on SIRT6 activity | Cellular and in vivo effects | Ref  |
|---------------|-----------|--------------------------|----------------------------|------|
| 11e 5-Cl-PZA  | ![Structure](https://example.com/structure.png) | $IC_{50} = 33.2 \mu M$ (deacetylation) | -                           | 150  |
| 14b BH3-HM3  | ![Structure](https://example.com/structure.png) | $IC_{50} = 8.1 \mu M$ (demethyls proliferation) | SIRT6 inhibition and increased TNFα fatty acylation in HEK293T cells. | 154  |
| 15f           | ![Structure](https://example.com/structure.png) | $IC_{50} = 0.219 \mu M$ (demethyls proliferation) | No effects, likely because of poor permeability. | 155  |
| 17a Catechin gallate | ![Structure](https://example.com/structure.png) | $IC_{50} = 2.50 \mu M$ (deacetylation) | -                           | 103, 104 |
| 19b OSS 128167 | ![Structure](https://example.com/structure.png) | $IC_{50} = 89 \mu M$ (deacetylation) | Augmented H3K9 acetylation and TNF-α secretion in BxPC3 cells. GLUT1 upregulation and increased glucose uptake in L6 rat myoblasts and BxPC3 cells. Improved glucose tolerance and reduced plasma levels of insulin, triglycerides, and cholesterol in a murine model of type 2 diabetes. Sensitization of MM to DNA-damaging chemotherapeutics. Decreased viability and proliferation inhibition of DLBCL cells. Reduced tumor growth and Ki-67 levels in mouse xenografts. | 79, 163, 164, 165 |
| 20b           | ![Structure](https://example.com/structure.png) | $IC_{50} = 37 \mu M$ (deacetylation) | Increased H3K9 acetylation in BxPC3. Augmented glucose uptake in L6 rat myoblasts and BxPC3 cells. Sensitization of BxPC3 cells to gemcitabine. Enhancement of olaparib anticancer activity in Capan-1 cells. | 166, 167 |
| 21b           | ![Structure](https://example.com/structure.png) | $IC_{50} = 22 \mu M$ (deacetylation) | Increased H3K9 acetylation and glucose uptake in PBMCs. Impaired TNF-α secretion and T lymphocyte proliferation. Sensitization of pancreatic cancer cells to gemcitabine. | 168 |
| 22a A127-CONHPI-B178 | ![Structure](https://example.com/structure.png) | $IC_{50} = 6.7 \mu M$ (demethyls proliferation) | Increase of DNA-damage markers and telomere-dysfunction induced foci in HUVECs. Reduction in TNF-α levels. | 169 |
| 23            | ![Structure](https://example.com/structure.png) | $IC_{50} = 4.93 \mu M$ (deacetylation) | Dose-dependent increase of H3K9 and H3K18 acetylation levels in BxPC3 cells. Increased GLUT-1 expression levels. Reduction of blood glucose content in a mouse model of type 2 diabetes. | 171 |
SIRT6 catalyzes the demyristoylation of TNF-α at Lys19 and Lys20, triggering its secretion during inflammatory response, while acetylated TNF-α is retained and finally degraded in lysosomes. In addition, TNF-α levels are positively regulated by NAD+ concentration, and SIRT6 was identified as the mediator of the increased translation efficiency of Tnf mRNA.

On the other hand, SIRT6 also exerts anti-inflammatory roles through negative regulation of NF-κB, and is supported by studies in macrophages where SIRT6 deletion promotes NF-κB activation and IL-6 production. Studies performed on SIRT6-knockout mice indicated a chronic liver inflammation and fibrosis. Moreover, SIRT6-deficient lymphocytes and myeloid-derived cells presented aberrant activation. Mechanistically, SIRT6 repressed the transcription of genes controlled by the oncogenic transcription factor c-JUN (Figure 3).

Sugar and Lipid Metabolism. SIRT6 is undoubtedly a multitasking protein, and beyond its involvement in DNA maintenance and cancer progression, its main function is probably the regulation of glucose and lipid metabolism. As outlined in the context of cancer, SIRT6 corepresses HIF-1α and deacetylates H3K9 at glycolytic genes promoters, thus channeling glucose catabolism from glycolysis toward more energy-efficient pathways (Figure 4). Indeed, SIRT6-knockout mice display increased glycolytic pathway associated with high glucose uptake, increased insulin signaling, and severe hypoglycemia as a compensatory response.

SIRT6 modulates glucose homeostasis also through control of gluconeogenesis. SIRT6 has been found to deacetylate the acetyltransferase general control nonderepressible 5 (GCN5), a regulator of cell cycle progression involved in the onset of different tumors, leading to an increased enzymatic activity. In the liver, increased GCN5 activity results in acetylated PGC-1α, thus leading to reduced gluconeogenesis gene expression, which prevents hyperglycemia in diabetic/obese mice. Another important transcription factor for gluconeogenesis is FoxO1, which activates the transcription of the rate-limiting gluconeogenesis enzymes glucose-6-phosphatase (G6PC) and phosphoenolpyruvate carboxykinase (PCK1). FoxO1 is deacetylated by SIRT6, triggering its nuclear export and reduced transcription of its target genes (Figure 3).

SIRT6 activity has also an effect on insulin signaling through downregulation of glucose transporters GLUT1 and GLUT4 and decreased phosphorylation of AKT, an important regulator of cellular glucose uptake. Mechanistically, SIRT6 is involved in the inactivation of AKT upstream proteins, including the insulin receptor substrates IRS1/2. Therefore, the absence of SIRT6 sensitizes the organism to insulin action, giving a complementary explanation to glycolytic gene suppression for the observed hypoglycemia in SIRT6-knockout mice.

In the case of lipid metabolism, SIRT6 reduces triglyceride synthesis and fatty-acid uptake while promoting β-oxidation, as indicated by mice-knockout studies. SIRT6 also contributes to keeping low the levels of LDL-C (Figure 3). Mechanistically, SIRT6 has been shown to decrease acetylation of the PPARα coactivator NCOA2, although it is not clear whether NCOA2 is a direct substrate of SIRT6 enzymatic activity. This determines activation of PPARα, a key transcription factor for hepatic β-oxidation genes. Furthermore, SIRT6 represses the expression of the proprotein convertase subtilisin/kexin type 9 (PCSK9), which controls the degradation of LDL-C in lysosomes (Figure 3).

Through interaction with FoxO3α, SIRT6 is recruited at the PCSK9 promoter, where it deacetylates H3K9 and H3K56, hence suppressing its transcription. SIRT6 also deacetylates H3K9 and H3K56 at the promoters of genes regulated by the sterol regulatory element-binding protein 1 and 2 (SREBP1/2). These transcriptional regulators activate transcription of lipogenic genes and are also directly controlled by SIRT6 at the transcriptional level through H3K56 deacetylation at promoters. Notably, micro-RNAs miR-33a and mi33b, which are expressed from the introns of SREBP1/2, are associated with repression of SIRT6 levels, contributing to a negative feedback loop in the SIRT6-SREBP1/2 axis. Another micro-RNA involved in SIRT6-mediated pathways is miR-122, the most abundant hepatic microRNA, which negatively regulates SIRT6 expression and is in turn negatively regulated by SIRT6 (Figure 3). In addition, while SIRT6 positively regulates genes involved in fatty-acid β-oxidation, miR-122 performs an opposite action.

The connection between SIRT6 activity and fatty-acid metabolism is fascinating given the evidence indicating that FFA are capable of increasing SIRT6 activity in vitro. Therefore, SIRT6 may act as a fatty-acid sensor that amplifies metabolic signals into epigenetic responses that affect crucial homeostatic mechanisms beyond metabolism itself; these
include all the pathways analyzed in this section such as genomic maintenance, immunity, cellular differentiation, and transformation.

■ PHARMACOLOGICAL MODULATION OF SIRT6

The implications of SIRT6 as a positive regulator of metabolism and aging, along with the discovery that the deacetylase activity may be enhanced by FFA, has stimulated research groups toward the development of SIRT6 activators (Table 1). On the other hand, given the dual role of SIRT6 in inflammation and cancer, inhibitors have also been developed (Table 2). The possibility of either activating or inhibiting SIRT6 in a context-dependent manner paves the way for personalized pharmacology. From a wider perspective, highly potent and selective SIRT6 modulators (both activators and inhibitors) allow the molecular details of its activity to be better scrutinized and further validate the enzyme as a pharmacological target.

In the following section, we will first discuss the most relevant SIRT6 activators followed by a detailed description of SIRT6 inhibitors.

SIRT6 Activators. As already mentioned, early studies on SIRT6 activity indicated that FFA containing 14 to 18 carbons (Figure 5, upper panel) stimulated SIRT6 activity. In particular, myristic acid (1a) increased deacetylase activity up to 10.8 times, with an EC$_{50}$ of 246 μM with a 35-fold increase in catalytic efficiency ($k_{cat}/K_m$ value, i.e., the ability of SIRT6 to capture a substrate for catalysis) at 400 μM, suggesting increased affinity of SIRT6 for the acetylated substrate. Oleic (1b) and linoleic acid (1c) displayed EC$_{50}$ values of 90 and 100 μM, yielding an increase in deacetylase activity of 5.8 and 6.8 times, respectively. In the same study, 1a was shown to act as a competitive inhibitor for demyristoylation, suggesting that the same hydrophobic pocket occupied by FFA during deacetylation is necessary to accommodate the long acyl chain of fatty-acid substrates for deacylation. These findings are the basis for the development of small-molecule SIRT6 activators.

Following the studies on FFA, it has been shown that myristoylethanolamide (MEA, 2a) and oleoylethanolamide (OEA, 2b), the ethanolamine derivatives of 1a and 1b, showed a 2-fold maximum activation of SIRT6 and EC$_{50}$ values of 7.5 and 3.1 μM, respectively. In the same study, 1b and 1c were tested, yielding SIRT6 maximum-fold activation of 4.6 and 3.7 along with EC$_{50}$ values of 89 and 230 μM, respectively. Rahnasto-Rilla et al. also evaluated the influence of the flavonoids luteolin (3a) and quercetin (3b) on SIRT6 activity. The skeleton of flavonoids consists of a benzene ring (A) fused with a heterocyclic pyran ring (C) presenting a further phenyl group (ring B) in position 2. All the compounds described here present hydroxyl groups on carbons 5 and 7 in ring A (Figure 5, lower panel). 3b belongs to the subclass of flavonols and are characterized by an oxidized pyran ring, bearing a carbonyl group in position 4 and an additional hydroxyl group in position 3. Differently, 3a is a flavan and lacks the hydroxyl group in position 3. Both compounds demonstrated a dose-dependent role, whereby they exert inhibitory activity at low concentrations with IC$_{50}$ values of 1.9 μM (3a) and 24 μM (3b) while increasing the deacetylase activity at higher concentrations. In particular, 3a showed a 6-fold maximum activation with an EC$_{50}$ of 270 μM, while 3b yielded 10-fold maximum activation and an EC$_{50}$ of 990 μM. Although the EC$_{50}$ values for these two flavonoids are very high and with scarce pharmacological relevance, these results suggest multiple binding sites for small molecules, which may interact with an inhibition site at low concentrations while inducing favorable conformational changes that activate the enzyme at higher concentrations.

Following these studies, further flavonoids were tested for their capability of altering SIRT6 enzymatic activity. The flavonol myricetin (3c) has the same structure of 3b with an
polar interactions are shown as dashed orange lines.

massive increase in activation e

fl

3b

α

−

H3 acetylation, associated with HAT activation. Moreover, compounds 3a–c have been reported to interfere with multiple bioassays, thus being classified among the pan assay interference compounds (PAINS) and suggesting caution in interpreting the results of biological studies on them.

Nonetheless, flavonoids could represent useful hit compounds for the development of SIRT6 activators thanks to the release of SIRT6–3b and SIRT6–3e cocrystal structures (PDB IDs: 6QCD and 6QCH, respectively). These structures indicated that 3b and 3e interact with SIRT6 at the distal end of the hydrophobic acyl-binding pocket, with surface contacts with the β6/α6 loop that caps this channel (Figure 6). In both cases, the catechol portion (ring B) is inserted in the acyl-binding pocket with the 4′-hydroxyl group forming a hydrogen bond with Pro62 backbone oxygen and with a conserved water molecule that in turn forms a hydrogen bond with the backbone oxygens of Ala53 and Ile61. Similarly, the 3′-hydroxyls of both molecules are hydrogen-bonded with another conserved water molecule that is in contact with the side-chain oxygen of Asp116.

In the case of 3b, the chromen-4-one moiety (rings A and C) forms hydrophobic contacts with Phe64/82/86, Val70/115, and Met136/157 (Figure 6). In particular, at a 312.5 μM concentration, the enzymatic activities of SIRT1/2/3/5 were 60–70% compared to the respective controls, while SIRT6 activity was about 150%. Given their polyphenolic nature, flavonoids are known to present pleiotropic activities and have been shown to inhibit a diverse subset of enzymes. Among others, the starch digestive enzyme α-glucosidase is inhibited by compounds 3a–c and 3e with IC₅₀ values in the low–mid μM range, while α-amylase was shown to be inhibited by 3a–c with IC₅₀ values of ~300 μM. Moreover, compounds 3a–c have been reported to inhibit topoisomerasases I and II and to affect the epigenetic regulation of transcription through inhibition of DNA methyltransferase 1 (DNMT1). 3b was also shown to suppress the activity of other epigenetic enzymes such as HDAC1 and the histone acetyltransferase (HAT) p300. In contrast, a different study indicated that 3b administration results in increased histone H3 acetylation, associated with HAT activation. Moreover, compounds 3a–c have been reported to interfere with multiple bioassays, thus being classified among the pan assay interference compounds (PAINS) and suggesting caution in interpreting the results of biological studies on them.

The crystal structures of SIRT6 in complex with 3b and 3e enable the identification of key features for ligand binding and, likely, could be exploited to develop new compounds containing only the hydroxyl groups essential for the interaction with the target, thus decreasing the polyphenolic character. Moreover, computational scaffold hopping approaches integrated...
with AI-driven drug discovery\textsuperscript{122} could allow the design of derivatives bearing a different core but retaining the moieties important for SIRT6 interaction. Overall, these strategies could enable molecules with increased specificity and potency to be obtained.

Another naturally occurring molecule showing SIRT6 activation is fucoidan (4), a heterogeneous sulfated polysaccharide present in brown algae. Its backbone consists of repeating (1→3) or (1→4) linked α-l-fucopyranose residues, in which some hydroxyl groups form sulfated esters (Figure 5, lower panel).\textsuperscript{123} The oversulfated fucoidan subtype extracted from Fucus vesiculosus displayed a 355-fold increase of SIRT6 activity at a 100 μg/mL concentration. In addition, when tested against other SIRTs (SIRT1/2/3), it did not display significant changes in activity, suggesting a specific action toward SIRT6. 4 was also able to activate SIRT6 acetylation toward H3K9 in vitro. According to the authors of the study, sulfate esters may play a central role in SIRT6–4 interaction and hence SIRT6 activation.\textsuperscript{123} However, the heterogeneity of the mixture, the polymeric nature of the compound, and the absence of kinetic data makes it difficult to compare this macromolecule to small molecules and to devise structure–activity relationships.

The first synthetic SIRT6 activator is the pyrrolo[1,2-a]quinoxaline derivative UB5039 (5, Figure 7a, upper panel), which exhibited an EC\textsubscript{50} of 38 μM and 3.5 maximum activation of SIRT6 in H3K9Ac peptide deacetylation assays.\textsuperscript{124} 5 showed specific binding on SIRT6, with no significant effects on basal SIRT1, 2, and 3 deacetylation activities. Notably, it stimulated SIRT5 desuccinylation activity (2-fold increase at 100 μM), the physiologically dominant activity of this enzyme. The 5–SIRT6 cocrystal (solved at 1.87 Å resolution, PDB ID: 5MF6) indicated a similar binding mode to 3b and 3e, with the compound occupying the exit of the acyl channel pocket and exposing the benzene moiety of the quinoxaline to solvent. The tricyclic portion of the molecule likely forms a methionine–aromatic ring interaction with Met136 along with weak hydrophobic interactions with Trp71, Phe82, Phe86, Ile185, and Met157. In addition, the pyridine nitrogen forms a hydrogen bond with the backbone carbonyl of Pro62 (Figure 7a, lower panel); this interaction represents a key anchoring point as a shift of the position of the nitrogen led to decreased SIRT6 affinity and activation. Comparison of this crystal structure with the cocrystal of SIRT6 and myristoylated peptide indicates that UBC5039 overlaps with the last seven carbons of the myristoyl chain. In addition, comparison with the SIRT6/ADP–ribose/3b cocrystal indicates that the compounds share a similar binding site. The 5 pyridine portion overlaps with 3b catechol moiety, and both engage in the key interaction with Pro62. In addition, the pyrrolo[1,2-a]quinoxaline moiety of 5 and the chromen-4-one are involved in similar hydrophobic interactions. One difference relies on the fact that 3b possesses a carbonyl group pointing toward Met136/157, which may impair optimal hydrophobic contacts between the aromatic ring and the methionine residues. Differently, the 5 tricyclic system is positioned in a privileged location for aromatic and hydrophobic interactions with Met136/157, thus explaining its higher potency compared to 3b. Although 5 did not display significant inhibition of SIRT6-mediated demyristoylation, as the binding affinity for the myristoylated peptide is much higher, addition of myristoylated peptide decreased 5 binding by an order of magnitude, thus indicating competition for the same binding site. Compound 5 was also tested using physiological substrates, such as full-length histones extracted from calf thymus and HeLa nucleosomes. In both cases, Western blot analysis indicated H3K18 deacetylation in the presence of 5.\textsuperscript{124} Follow-up studies indicated that 5 causes SIRT6 activation in a different subset of cancer cell lines, including NSCLC, colon and epithelial cervix carcinoma, and fibrosarcoma. 5-mediated SIRT6 activation led to decreased H3K9 and H3K56 acetylation and autophagy-related cell death.\textsuperscript{125} This study represents the first evidence of in-cell small-molecule-mediated SIRT6 activation, suggesting a potential therapeutic exploitation of this activity.

A compound screening for drug repurposing recently identified the HMG-CoA reductase inhibitor fluvastatin (6, Figure 7b, upper panel), already approved for hypercholesterolemia treatment, as a SIRT6 activator.\textsuperscript{126} 6 showed an EC\textsubscript{50} = 7.1 μM and decreased H3K9 and H3K56 acetylation in HepG2 cell lines. This effect was accompanied by increased nuclear translocation of SIRT6. In addition, 6 treatment increased levels...
of phosphorylated AMPKα, which in turn promoted SREBP1 phosphorylation at Ser372. In addition, cleaved SREBP1 was negatively regulated. These results are in line with previous reports suggesting that SIRT6 overexpression suppresses SREBP1/2 through the AMPK pathway.99 Interestingly, a subsequent study found a much higher EC_{50} (>250 μM) for 6-mediated SIRT6 activation, though it could reach 3.5-fold maximum activation at 1 mM.127 Compound 6 also displayed weak inhibition of SIRT1/2/3, while it did not affect SIRT7 activity. Nonetheless, the authors managed to cocrystallize 6 with the N-terminally truncated SIRT6 (13–308) and ADP–ribose and solved the structure at 2.46 Å (PDB ID: 6ZU4, Figure 7B, lower panel). 6 interacts with SIRT6 at the exit of its acyl channel in its acid form, rather than lactone, forming a hydrogen bond with Trp188 through its carbonyl group. In addition, the fluorophenyl and isopropyl residues point toward the channel exit, while the heptenonic acid moiety interacts with a surface formed by Lys15, Thr71, and Glu74. The indole moiety has a similar positioning of the pyridine ring of 7a as it is oriented toward the hydrophobic pocket formed by Phe64/82/86, Ile61, Pro62, and Met136. However, bulky substituents, such as the isopropyl and fluorophenyl groups, obstruct the entrance in the pocket, thereby impairing the key polar interactions with the backbone oxygen of Pro62 seen with 5 and other ligands. In summary, the authors of this study suggest that the initially measured low EC_{50} may be a result of an assay artifact and that the reported cellular effects may be due to an indirect action of 6. Nevertheless, the elucidation of the 6 binding mode aids the development of modulators possessing the same core scaffold, but different substituents, in order to maximize polar interactions.

Virtual screening followed by in vitro evaluation led to the discovery of novel and cellularly active SIRT6 activator, the 2-prismene-4-(phenylsulfonamido)benzenesulfonamide derivative MDL-800 (7a, Figure 8A).128 7a displayed an EC_{50} value of 10.3 μM, enhancing SIRT6 activity by more than 22 times (at 100 μM), using a synthetic acetylated peptide (RHKK-ac-AMC) as a substrate. When tested on SIRT6 using the H3K9Ac peptide (KQGTK-ac-STGGWV), 7a exhibited 18-fold maximal SIRT6 activation. In addition, 7a increased the deacetylation of H3K9 and H3K56 on HeLa-extracted nucleosome substrates in a dose-dependent manner. 7a did not display any effect on the enzymatic activities of SIRT1/3/4 and HDAC1–11 at concentrations up to 50 or 100 μM. It displayed weak inhibition of SIRT2 (IC_{50} = 100.4 μM) and weak activation of SIRT5 (IC_{50} = 104.6 μM) and SIRT7 (IC_{50} = 187.1 μM). Since the IC_{50}/EC_{50} values are 10 times (or more) greater than SIRT6 EC_{50}, the compound is considered selective. The analogue MDL-801 (7b), in which the methyl carboxylate ester in position 2 of the central benzenesulfonamide ring is replaced by a carboxylic group (Figure 8A), exhibited overlapping SIRT6 activation features with an EC_{50} = 5.7 μM. However, while 7a was highly cell permeable and accumulated in cells, 7b had poor cellular permeability and a high efflux ratio. Therefore, the only compound tested for cellular activity was 7a. This molecule caused a dose-dependent decrease of H3K9Ac and H3K56Ac in HCC cells (specifically Bel7405, PLC/PRF/5, and Bel7402 cell lines), leading to inhibition of their proliferation through SIRT6-mediated cell cycle arrest. In particular, the observed IC_{50} for cell growth (IC_{50, growth}) was between 18.6 and 24 μM, depending on the cell line. These results were confirmed in mouse xenograft models, where 7a suppressed HCC tumor growth through SIRT6 activation. A recent investigation indicated that 7a inhibits the proliferation of 12 NSCLC cell lines in a dose-dependent manner and caused cell cycle arrest at the G_{1}/G_{0} phase in NSCLC HCC827 and PC9 cells, consistent with studies indicating the role of SIRT6 in cell cycle regulation.16,70 Notably, it exhibited synergistic activity with epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) in osimertinib-resistant HCC827 and PC9 cells and in patient-derived primary tumor cells. Moreover, 7a suppressed tumor growth in HCC827 cell-derived xenograft nude mice and caused H3 deacetylation and downregulation of p-MEK and p-ERK in tumor tissues.129

Huang et al. solved the cocrystal structure of the complex formed by SIRT6, ADP–ribose, H3K9 myristoylated peptide, and 7b (PDB ID: SY2F, Figure 8B).128 Given the structural similarities between 7b and 7a, the observed features are likely shared between the two compounds. Interestingly, 7b appeared to interact with SIRT6 in a unique pocket, distinct from the binding site of 3b, 3e, 5, and 6 located in the acyl-binding hydrophobic channel. Indeed, 7b was shown to interact with a surface-exposed distal region defined by the N-terminal residues 1–7, Val70, Glu74, Phe82, Phe86, Val153, and Met157. The 3,5-dichlorobenzene moiety of 7b is involved in weak polar interactions with Asn40, Val70, and Glu74 and engages π-stacking interactions with Phe82 (Figure 8B). The central 2-carboxybenzenesulfonamide ring is also involved in π-stacking interactions with Phe86, whose importance was confirmed by single-residue mutation experiments showing decreased potency of both 7a and 7b toward SIRT6-F86A.128 However, a recent report from You and Steegborn argued that the observed electron density could be attributed to a molecule of morpholinoethanesulfonic acid (MES), used as crystallization buffer, rather than 7b.130 Therefore, they determined new crystal structures for SIRT6 in complex with 7b. They solved the cocrystal of N-terminal truncated SIRT6_{13–308} in complex with ADP–ribose and 7b (PDB ID: 6XV1) as well as in the absence of 7b (PDB ID: 6XUY). Similarly, they solved the structure for SIRT6_{13–308} (comprising the N-terminus) in complex with ADP–ribose and 7b (PDB ID: 6XVG, Figure 8C), along with a reference structure without the activator (PDB ID: 6XV6).130 These structures indicate a different binding mode for 7b, which does not bind at the distal end of the acyl-binding hydrophobic channel but in the same region as the previously described activators 3b, 3e, and 5. In both SIRT6_{13–308}–7b and SIRT6_{13–308}–7b structures, the activator engages in extensive hydrophobic interactions, the central 2-carboxybenzenesulfonamide is packed between Val70, Trp71, and Met157, and the 5-bromo-4-fluoro-2-methylaniline portion interacts with Phe64, Val70, Phe82, Phe86, and Val115. In addition, bromine forms a halogen bond with the backbone amide oxygen of Pro62, which has been shown to be a key residue for small-molecule interactions with SIRT6 (Figure 8C). Notably, the interaction with Pro62 is missing in the binding mode illustrated by Huang et al.128 The 3,5-dichlorobenzene moiety is less defined and seems to be largely solvent-exposed. Hence, the structures from the two groups display rather different binding modes for 7b, whose orientations within SIRT6 are perpendicular to each other in the two studies. In response to this report, Huang et al. crystallized SIRT6 with and without 7b using the same conditions as in their original publication (PDB ID: 7CLO for SIRT6 in complex with ADP–ribose and H3K9 myristoyl peptide; PDB ID: 7CL1 for SIRT6 in complex with ADP–ribose, H3K9 myristoyl peptide, and MDL-801).128,131 They showed that, in the absence of 7b, the buffer molecule MES does
not fit properly the originally proposed ligand-binding pocket. In addition, the newly solved cocrystal in the presence of 7b, although possessing lower overall resolution (3.2 Å for 7CL1 vs 2.53 Å for 5Y2F), has better electron density for the activator and confirms their initial findings. Importantly, Huang et al. crystallized SIRT6 in the presence of the H3K9 myristoyl peptide, which is absent in the crystallization mixture of You and Steegborn. Overall, the observed differences in the 7b binding mode may be ascribed to the presence of the substrate, which influences the interaction between the small molecule and SIRT6. Indeed, the crystal structure represents just a conformational state of the protein, whose conformational dynamics can be altered by the presence of ligands, thereby leading to alteration of key interactions between a small molecule and their target. Hence, structures of SIRT6–7b from both groups may be equally valid and represent two different states of the protein, regulated by the presence of substrate. Nonetheless, further experiments, including the structure of 7b in the presence of acetylated substrate, may help to further clarify this controversy.

The replacement of the methyl carboxylate with an N-methyl-3-methylphenoxy at the C3 position of the central benzene ring of 7a led to compound MDL-811 (7c, Figure 8A) with improved activity (EC_{50} = 5.7 μM) and bioavailability in C57BL/6J mice (F_{MDL-811} = 71.33% vs F_{MDL-800} = 92.96%). The improved activity may be explained by the higher number of interactions that the N-methyl-3-methylphenoxy moiety can establish. Indeed, it presents an exposed oxygen that can act as a hydrogen bond acceptor and a methyl group potentially involved in hydrophobic interactions. The compound enhanced deacetylation of H3K9, H3K18, and H3K56Ac levels in different CRC cell lines and patient-derived xenograft (CDX and PDX, respectively) models as well as in a spontaneous CRC mouse line-derived and patient-derived organoids and showed antitumor efficacy in cell- and patient-derived xenograft (CDX and PDX, respectively) models as well as in a spontaneous CRC mouse model. Mechanistically, cytochrome P450 family 24 subfamily A member 1 (CYP24A1), which had been previously shown to be an activator of SIRT6, strongly stimulated SIRT6 deacetylase activity in a time-dependent manner. In line with this, 7c suppressed CRC growth in patient-derived organoids and showed antitumor efficacy in cell-line-derived and patient-derived xenograft (CDX and PDX, respectively) models as well as in a spontaneous CRC mouse model. Mechanistically, cytochrome P450 family 24 subfamily A member 1 (CYP24A1), which had been previously shown to be an activator of SIRT6, strongly stimulated SIRT6 deacetylase activity in a time-dependent manner.

Activity-based screening of lipid-like molecules led to the identification of 8a (Figure 8A), a compound consisting of a 4-carboxyphthalimide conjugated to an N-(2-chlorophenyl)2,5-dichlorobenzamide. 8a displayed an EC_{50} = 97 μM and 17 maximum-fold activation of SIRT6 deacetylase activity. In addition, it displayed selectivity over SIRT1–3 and SIRT5. Therefore, 8a represented an ideal lead compound for the development of selective SIRT6 activators. Removal of chlorine atoms from either the 2,5-dichlorophenyl moiety or trichlorobenzoyl group at the aniline nitrogen led to CLSD (8b), which showed 7-fold increased potency over 8a, with an EC_{50} = 15.5 μM. Notably, the methyl ester of 8b did not show any activity. The data obtained from the development of 8b indicate that electron-drawing groups on the aromatic rings are crucial for SIRT6 activation in this series of molecules. In addition, the anionic headgroup (the carboxylic acid) is also essential for activity, and it is probably involved in hydrogen bond interactions. The maximum-fold activation of 8b was measured in terms of the k_{cat}/k_{m} ratio, which was ~50 under steady-state conditions. 8b displayed competitive inhibition of demethylation (K_i = 13.4 μM), suggesting occupation of the acyl-binding pocket, although structural data are missing. 8b also stimulated SIRT6 deacetylase activity in a time-dependent manner.

A recent study that evaluated the influence of the FDA-approved DNA hypomethylating agents (DHAs) on Sirtuin family members showed that the nucleoside analogues 5-azacytidine (5AC, 9a), decitabine (DAC, 9b), and zebularine (9c) increased SIRT6 enzymatic activity (Figure 9, middle panel). 9a and 9b increased SIRT6 activity after 12, 24, and 48 h of incubation at 0.25 and 0.5 μM; albeit, no dose-dependency was observed. Moreover, while the maximum activation (1.3-fold activation) for 9a was observed after 48 h, 9b exhibited 1.5-fold activation after 12 h, followed by a decrease in activation efficiency at 24 and 48 h. 9c could also activate SIRT6 deacetylase activity, although at higher concentrations (0.5 and 1 μM), with 1.4 maximum-fold activation observed after 48 h of

Figure 9. Further synthetic SIRT6 activators.
incubation. In addition, both 9a and 9c (but not 9b) reduced the enzymatic activity of SIRT1, while the activity of SIRT2, SIRT3, and SIRT5 was not affected by any of these compounds. Although these data indicate that these compounds activate SIRT6, the lack of dose dependency suggests that they have been tested far below their EC_{50}; hence, the maximum activation values presented here should be taken cautiously. In line with these results, U937 leukemia cells treated with 0.5 μM 9b for 24 and 48 h displayed decreased levels of H3K9Ac and H3K56Ac, according to Western blot experiments. Further ChIP-Seq analysis of bone marrow cells derived from six AML patients and 48 h displayed decreased levels of H3K9Ac and H3K56Ac, establishment hydrophobic interactions with Tyr5, Val70, Phe82, Pro62, and Pro80, and one of its benzene rings forms π−π interactions with Phe86. According to this model, although located in a similar region, compound 10b binds SIRT6 more toward the end of the hydrophobic channel compared to 9a, which may justify 10b-mediated enhancement of SIRT6 deacylation activity. Compound 10b suppressed the proliferation and caused cell cycle arrest in the G2 phase of PANc-1 and BXPC-3 PDAC cell lines. Cellular thermal shift assay (CETSA) performed in intact cells confirmed that 10b (at 25 μM concentration) interacts with SIRT6 in cells. In addition, 10b exhibited antitumor activity in a human pancreatic xenograft mouse model associated with a decrease of H3K9 acetylation levels. A preliminary study in male Sprague-Dawley rats also indicated a promising pharmacokinetic profile, although the bioavailability was only 4%. Although 10b with its low micromolar EC_{50} values is a promising lead compound, we cannot exclude that the effects observed in cells and in vivo are related to interactions with off-target proteins beyond SIRT6.

Therefore, further functional and target engagement assays such as mass-spectrometry-based thermal profiling, fluorescence resonance energy transfer imaging (FRET) probes, and affinity-based protein profiling (ABPP) seem necessary to clarify this point. Moreover, genetic studies alone and in combination with compound treatment in both cellular and animal PDAC models would be required to confirm the causal link between the observed phenotypes and SIRT6 activation and to conclusively assess the therapeutic potential of 10b in this tumor context.

**SIRT6 Inhibitors.** Given the double-faced involvement of SIRT6 in cancer and inflammation, inhibition of SIRT6 in specific contexts may represent a successful strategy for cancer management. Indeed, inhibitors may target different SIRT6-mediated pathways that contribute to cancer progression such as DNA repair mechanisms, cell differentiation inhibition, and inflammatory response (Table 2).

Nicotinamide (11a, Figure 10) is one of the products of the sirtuin-mediated deacylation reaction and may act as a weak product inhibitor of SIRT6 without subclass specificity. 11a has been validated as a SIRT6 deacylation inhibitor through two different assays using H3K9 myristoyl peptides: an HPLC assay yielded an IC_{50} = 153 μM; similarly in a fluorogenic assay, 11a displayed an IC_{50} = 184 μM. In a subsequent study, 11a displayed an IC_{50} for a demyristoylation reaction of 73 μM while...
showing increased inhibitory potency toward deacylation of H3K9 decanoyl peptide (IC$_{50}$ = 45 μM) and lower potency using H3K9 hexanoyl peptide as a substrate (IC$_{50}$ = 184 μM).39

Based on the 11a analogue pyrazinamide (PZA), Bolivar et al. developed two derivatives with improved SIRT6 inhibition activity (Figure 10): 5-MeO-PZA (11b, IC$_{50}$ = 40.4 μM) and 5-Cl-PZA (11c, IC$_{50}$ = 33.2 μM). Remarkably, these compounds did not show NAD$^+$ competition, hence indicating a different mechanism of action from 11a.150 11c was reported to be active toward SIRT1, but not SIRT2/3, while 11b was not evaluated against SIRT1−3. Nonetheless, selectivity against other SIRTs and HDACs need to be ascertained.

ADP−ribose (12, Figure 10) also inhibits SIRT6 activity and showed higher potency than 11a with IC$_{50}$ values of 74 μM (deoctanoylation) and 89 μM (demyristoylation), compared to values of 150 and 120 μM, respectively, for 11a.151

Another class of inhibitors directly related to the SIRT6 enzymatic mechanism are N$^\varepsilon$-thioacyl lysine peptides, which cause a stall of the catalysis after the nucleophilic attack of the (thio)carbonyl group to the C1′ of nicotinamide-bound ribose that happens in the first step of the catalytic mechanism.152 Early reports following the thioacyl peptide strategy led to N$^\varepsilon$-thioacyl lysine pentapeptides 13a and 13b (Figure 11, upper panel) showing IC$_{50}$ values toward SIRT6 deacytase activity of 78 and 47 μM, respectively.153 These data indicate that replacement of a His residue with an Ala residue improves inhibitor activity. Both compounds inhibit SIRT1/2 with higher potency compared to SIRT6. Indeed, they both abolish SIRT1/2 almost completely at a 200 μM concentration, while the inhibition of SIRT6 was 62%
In successful example of synthetic SIRT6 inhibitors, inhibition in the low micromolar range with IC50 values for central being the most potent. A later study described the development of thiomyristoyl peptides designed on the basis of SIRT6 natural substrates. In particular, compounds BHJH-TM1 (14a), BHJH-TM3 (14b), and BH-TM4 (14c) (Figure 11, middle panel) displayed SIRT6 inhibition in the low micromolar range with IC50 values for demyristoylation of 2.8, 8.1, and 1.7 μM, respectively. These compounds were based on TNFα-K20, TNFα-K19, and H3K9 peptides, respectively. All three peptides were active against SIRT1/2/3, with IC50 values between 2.3 and 8.0 μM for all the isoforms, thus indicating a lack of selectivity and a mixed mode of action. Interestingly, they all displayed SIRT6 inhibition and increased TNFα fatty acylation in HEK293T cells with 14b being the most potent.

More recently, cyclic pentapeptides (15a–f) harboring a central N'-dodecyl- or N'-myristoyl-thiocarbamoyl-lysine (Figure 11, middle and lower panels) showed inhibitory activity toward SIRT6 in the nanomolar range (IC50 (15a) = 256 nM, IC50 (15b) = 282 nM, IC50 (15c) = 368 nM, IC50 (15d) = 319 nM, IC50 (15e) = 495 nM, IC50 (15f) = 319 nM). Compounds 15a–e had comparable IC50 values for SIRT1, while 15f an IC50 toward SIRT1 2.3 higher compared to SIRT6. Compounds 15e and 15f, bearing the same macrocycle bridging unit, were also tested against SIRT2 and SIRT3. Compound 15e showed moderate selectivity over SIRT2 and SIRT3 (~2.9-fold and ~1.5-fold, respectively), while 15f exhibited high selectivity over the two isoforms (20-fold and 11-fold, respectively). Finally, 15f was tested against SIRT5, where the results indicated that the molecule is substantially inactive towards this enzyme (IC50 > 300 μM). This analysis suggests that the only selective SIRT6 inhibitor is 15f. Despite that, 15f was not able to inhibit SIRT6 inside the human pancreatic cancer BxPC3 cells, likely because of poor cellular permeability given its peptide nature and high molecular weight. Nonetheless, these peptides represent valuable lead compounds for the development of peptidomimetics inhibiting SIRT6.

Recently, Sociali et al. developed a lysine-based compound targeting SIRT6 deacetylase and deacylase activities (16, Figure 11, lower panel). This molecule consists of a lysine residue whereby the N'-amine group is protected with an acetyl group, while the carboxy group is coupled with a 12-carbon alkyl chain amine. This compound inhibited SIRT6 deacetylation (IC50 = 95 μM) without isoform specificity, as it inhibited also SIRT1 and SIRT2 with comparable potency (IC50 = 51 and 102 μM, respectively). Remarkably, compound 16 behaved as a deacetylation activator showing 52% activation of demyristoylation (EC50 = 70 μM) and 80% activation of depalmitoylation at 100 μM while still acting as an inhibitor for SIRT1/2 deacylation (IC50 (SIRT1) = 157 μM, IC50 (SIRT2) = 177 μM). 16 displayed competitive inhibition toward acetylated peptide, but not NAD+, and increased H3K9 acetylation in the MCF-7 breast cancer cell line. Moreover, the activities of key glycolysis enzymes were increased, in line with SIRT6 involvement in downregulation of glycolytic enzymes, and TNF-α secretion was reduced, consistently with the ability of SIRT6 to trigger TNF-α secretion. The results obtained in this study are rather surprising in light of the evidence reported by Feldman et al. that FFAs determine enhancement of deacetylation activity and inhibition of deacylation. Based on in silico data, the authors speculate that the acetyl moiety bound to the Cα amine group may mimic the acetylated substrate, being close to NAD+, in agreement with the observed competition with acetylated substrate and not with NAD+.

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evidence is necessary to clarify the binding mode and account for the differential SIRT6 modulation profile.

Interestingly, the 3b derivatives (−)-catechin gallate (17a) and (−)-gallocatechin gallate (17b) displayed inhibition of SIRT6-mediated deacetylation in the low micromolar range (IC50 (17a) = 2.5 μM; IC50 (17b) = 5.4 μM).103 The epimers of compounds 17a and 17b, (−)-epicatechin gallate (17c) and (−)-epigallocatechin gallate (17d), displayed lower activity toward SIRT6, with ∼60 and ∼40% inhibition at 100 μM, respectively, compared to ∼85−90% inhibition of 17a−b at the same concentration. Structurally, these compounds differ from 3b in ring C, which is reduced and presents a 3,4,5-trihydroxybenzoyl substitution. The 17a–SIRT6 cocrystal (PDB ID: 6QCJ, Figure 12A) indicated that the inhibitor shares the same binding site as 3b with identical conformations of the catechol groups, while the chromen-4-one of 17a was rotated to accommodate the bulky trihydroxybenzoyl moiety. Ring C interacts with Trp71 of the acyl channel exit, and the trihydroxybenzoyl portion forms hydrophobic interactions with the other side of the channel and a hydrogen bond with the backbone of Gly155. It appears that the main difference between 3b-derived activators and inhibitors consists of the presence of the bulky substituent on ring C and consequent tilted position of the chroman, which is saturated in inhibitors 17a,b. This is supported by the fact that the orientation of the pyrrole[1,2-α]quinoline of the SIRT6 activator S is similar to the ring C of 3b derivatives, rather than 17a. Nonetheless, these compounds were not tested against other SIRTs or HDAC isoforms, so their selectivity needs to be further investigated. In addition, given their polyphenolic structure, both compounds very likely display pleiotropic off-target effects, as previously described for compounds 3a−e. Indeed, 17a−b also inhibit α-glucosidase,157 while inhibition of topoisomerases has been widely reported for the 17b epimer 17d.158,159 This compound also exhibited dual activity toward SIRT3, acting as either an activator or inhibitor depending on the cellular context.160 In addition, 17d inhibits DNMT1113,114 and different HAT enzymes such as p300, CBP, PCAF, and Tip60.86,87 Even though 17a−b have poor specificity, the availability of the SIRT6–17a cocrystal structure could be exploited by medicinal chemists for drug design, as previously mentioned in the case of activators 3b and 3e.

Trichostatin A (18, TSA), a hydroxamate derivative known for its nanomolar inhibitory activity of class I and II HDACs given its zinc-chelating properties, was recently found to inhibit SIRT6.161 Though no IC50 was calculated, the Kᵢ values for 18-mediated SIRT6 deacetylation were 2.02 μM when using H3K9Ac peptide and 4.62 μM when using p53K382Ac peptide. No inhibitory activity was observed against SIRT1−3 and SIRT5 up to a 50 μM concentration. Kinetic analysis indicated competitive inhibition toward the acetylated peptide but not NAD+. The crystal structure of the SIRT6/ADP-ribose/18 complex (PDB ID: 6HOY, Figure 12B) indicated the binding of 18 to the acyl channel extension of SIRT6, explaining its isoform specificity.162 The hydroxamate moiety of 18 engages in polar
interactions, including a water-mediated hydrogen bond between 18 nitrogen and the backbone oxygens of Ile 61 and Pro62. The carbonyl group is involved in hydrogen bonds with the backbone nitrogen of Val115 and Asp116. In addition, the 18 hydroxyl moiety acts as a hydrogen bond donor in its interaction with the side chains of Asn114 and Asp116 (Figure 12B). The 18 hydroxamate group mimics 11a interactions, as confirmed by a competition binding assay in the presence of 11a. Since no 18/NAD\(^{+}\) competition was observed in activity assays, the authors propose a mechanism whereby 18 interacts with the 11a binding region following the release of the 11a moiety from NAD\(^{+}\). They also argue that the reported acetylated peptide competition is caused indirectly, by inducing conformational changes leading to clashes with the acylated substrate.

Beyond drug repurposing, the first synthetic small-molecule compounds displaying SIRT6 inhibition were identified by Parenti et al. following an in silico screening.\(^{163}\) This approach led to the discovery of the derivatives 19a–c (Figure 13) possessing IC\(_{50}\) values of 106, 89, and 181 \(\mu \text{M}\), respectively. Among them, compounds 19b (subsequently named OSS_128167) and 19c displayed selectivity over SIRT1 and SIRT2 (IC\(_{50}\) values 8.44 to 19.15 times higher, while 19a was mildly selective over SIRT1 (IC\(_{50}\) = 314 \(\mu \text{M}\)) but not over SIRT2 (IC\(_{50}\) = 114 \(\mu \text{M}\)). The three compounds increased H3K9 acetylation in BxPC3 cells and induced GLUT1 upregulation and consequent augmented glucose uptake in L6 rat myoblasts and BxPC3 cells. This is consistent with reports indicating the role of SIRT6 in GLUT1 downregulation.\(^{36}\) Furthermore, the compounds were able to reduce TNF-\(\alpha\) secretion. This study shows that small-molecule-mediated SIRT6 inhibition mimics the effects of SIRT6 knockdown. When tested in a murine model of type 2 diabetes, 19a improved glucose tolerance and reduced plasma levels of insulin, triglycerides, and cholesterol.\(^{164}\) Remarkably, 19b reduced the recruitment of SIRT6 to DNA-damage locations and sensitized primary MM cells, along with melphalan- and doxorubicin-resistant MM cell lines, to DNA-damaging chemotherapeutics.\(^{165}\) Compound 19b also decreased the viability of DLBCL cells, usually displaying SIRT6 overexpression, and inhibited their proliferation in a time- and dose-dependent fashion, through induction of apoptosis and cell cycle arrest at G2/M phase. When tested in a mouse xenograft model with human DLBCL cells, 19b reduced tumor growth and decreased the levels of the proliferative marker Ki-67.\(^{79}\) Nevertheless, these reports lack of target engagement studies demonstrating that 19b does bind to SIRT6 at least in the cellular context. Hence, the observed in vitro phenotype may be a consequence of off-target effects, particularly considering the weak in vivo potency of 19b. To shed light on this, cellular and in vivo target engagement studies should be performed.\(^{139–143}\) Moreover, the comparison between the phenotypes induced by 19b treatment, by SIRT6 gene knockdown, and by a combination of the two should also be carried out to clarify the mechanism of action\(^{144,145}\) and unambiguously link the observed anticancer effects to SIRT6 inhibition.

Optimization of compound 19a led to the quinazolinedione derivatives 20a–c (Figure 13, left).\(^{166}\) Compounds 20a and 20b are characterized by different substituents on the sulphonamide residue; in addition to this, in compound 20c, the nitrogen atoms of the quinazolinedione core are methylated. These substitutions led to improved SIRT6 inhibition (IC\(_{50}\) (20a) = 60 \(\mu \text{M}\); IC\(_{50}\) (20b) = 37 \(\mu \text{M}\); IC\(_{50}\) (20c) = 49 \(\mu \text{M}\)). Compound 20a was slightly selective over SIRT1 and SIRT2 with IC\(_{50}\) values of 238 and 159 \(\mu \text{M}\), respectively. Compounds 20b–c exhibited good selectivity over SIRT1 (IC\(_{50}\) values were 11 and 133 times higher, respectively) and low selectivity over SIRT2 (2.30-fold and 4.94-fold, respectively), although the activity against other isoforms remains to be tested. It appears that removal of oxygen in the sulphonamide side-chain and the extension of the aliphatic spacer between the aromatic groups (see 20b) improves the inhibitory efficiency of these derivatives. In addition, the simultaneous oxygen removal from the side chain and methylation of the quinazolinedione nitrogens increases isoform specificity. These derivatives increased H3K9 acetylation in BxPC3, but only compounds 20b and 20c caused increased glucose uptake in L6 rat myoblasts and BxPC3 cells. Remarkably, 20a and 20b were able to sensitize BxPC3 cells to the chemotherapeutic gemcitabine. Compound 20c was not evaluated in vivo, since it was found to be cytotoxic at a concentration close to its IC\(_{50}\) (30 \(\mu \text{M}\)). Compounds 19a and 20b were found to effectively enhance the anticancer activity of the PARP inhibitor olaparib in Capan-1 cells (a BRCA2-deficient pancreatic cancer cell line). These observations are consistent with previous findings suggesting that SIRT6 knockdown improves the efficacy of chemotherapeutics.\(^{167}\)

The salicylate derivative 19b was further optimized yielding the highly selective SIRT6 inhibitors 21a–c (Figure 13, right). Compound 21a is an analogue of 19b, in which the furan-2-carboxamide moiety is shifted from 3’ to 4’. Compound 21b presents the furan-2-carboxamide at the same position as 21a but has the hydroxyl and carboxylic groups swapped with each other. 21a and 21b have IC\(_{50}\) values of 34 and 22 \(\mu \text{M}\), respectively. These data indicate that the presence of the furan-2-carboxamide at para position massively increases the SIRT6 inhibitory activity, while the swap of hydroxyl and carboxylic groups leads to only a slight improvement of the inhibition. In compound 21c, a pthalimide moiety replaces the furan-2-carboxamide in 3’, while the carboxylic and hydroxyl groups are in positions 2 and 4, respectively. These modifications furnished a compound with slightly improved inhibitory efficacy, having an IC\(_{50}\) of 20 \(\mu \text{M}\).\(^{166}\) All compounds displayed selectivity over SIRT1 and SIRT2 (IC\(_{50}\) values between 13 and 27 times higher), although the selectivity over other SIRT isoforms needs to be evaluated. Compounds 21a and 21b increased H3K9 acetylation and glucose uptake in human peripheral blood mononuclear cells (PBMCs), in line with previous studies and with the roles of SIRT6 in cell homeostasis. Conversely, compound 21c did not show any effect in cell-based assays, probably due to a lack of cell permeability. Compounds 21a and 21b also impaired TNF-\(\alpha\) secretion and sensitized pancreatic cancer cells to gemcitabine. Compound 21b also presented antiproliferative properties in PBMCs.

Compound screening based on a DNA-encoded library designed for NAD\(^{-}\)-binding pockets led to the identification of two SIRT6 inhibitors with a 5-aminocarbonyl-uracil core (Figure 13, lower panel): A127-(CONHPr)-B178 (22a) and A127-(CONHMe)-B178 (22b). Both molecules were evaluated in a demethylst-overlay assay and displayed IC\(_{50}\) values of 6.7 and 9.2 \(\mu \text{M}\), respectively.\(^{169}\) Compound 22a was selective over other SIRTs, as inhibition of SIRT1–3, SIRT5, and SIRT7 was less than 10% at 10 \(\mu \text{M}\) and was stable in serum after 72 h. It caused an increase of DNA-damage markers and telomere-dysfunction-induced foci in primary human umbilical venous endothelial cells (HUVECs), like what was observed following SIRT6 knockdown.\(^{170}\) Similarly to other SIRT6 inhibitors, 22a caused a dose-dependent decrease in the TNF-\(\alpha\) levels.
Recently, a series of 1-phenylpiperazine derivatives have been reported as a SIRT6 inhibitors. Among them, 5-(4-methylpiperazin-1-yl)-2-nitroaniline (23, Figure 13, lower panel) displayed an IC₅₀ of 4.93 μM in a peptide deacetylation assay and showed no activity against SIRT1–3 and HDAC1–11 up to 200 μM concentration.⁷¹ When tested in BxPC-3 cells, compound 23 augmented the level of both H3K9 and H3K18 acetylation in a dose-dependent manner and increased GLUT1 expression levels. In addition, it reduced the blood glucose content in a mouse model of type 2 diabetes, thus demonstrating promising lead-like properties.

**CONCLUSIONS**

Mounting evidence supports the critical roles of SIRT6 in multiple processes regulating both physiological and pathological states. Although SIRT6 shares mechanistic features with other SIRTs, it differs from them, as it greatly depends on FFA activation to increase the efficiency of its enzymatic activities.²⁰,²² Through its multiple enzymatic activities, SIRT6 finely regulates not only genome maintenance and DNA repair but also stem cell differentiation, metabolism, and aging. The involvement of SIRT6 in these key processes may explain its dual role in cancer. For instance, DNA repair promotion may help evasion from tumorigenic transformation at early phases of cancer. On the other hand, the same mechanism may facilitate cancer progression at later stages or decrease the effectiveness of cytotoxic drug chemotherapy. It is worth noticing that the upregulation of SIRT6 in certain types of cancers⁷⁶–⁷⁹ may be representative of a compensatory effect rather than the cause itself of tumor initiation and/or progression.⁷⁶

SIRT6 also regulates crucial proteins involved in sugar homeostasis, as it promotes the expression of glycolytic genes,⁵⁹,⁶⁰ suppressing gluconeogenesis and increasing insulin secretion, hence having a favorable role in diabetes. The downregulation of glycolytic genes also acts as a tumor-suppressor pathway, since it suppresses the Warburg effect.⁵⁸ SIRT6 also regulates fat metabolism by reducing LDL-cholesterol levels⁶⁵ and triglyceride synthesis as well as promoting fatty-acid β-oxidation,⁶⁶ being a key player in obesity prevention. Like the double-faced role in cancer, SIRT6 has contrasting actions in the regulation of inflammation.⁴⁰,⁴⁴,⁸⁵

Although the growing knowledge about SIRT6 biology has been uncovering multifaceted functions in human diseases, the discovery of potent and selective SIRT6 modulators is at its infancy. The notion that FFAs increase the deacetylation efficiency of SIRT6 led to the investigation and discovery of the first SIRT6 activators. Initial hit compounds were derived from simple modifications of fatty acids, such as the ethanolamides 2a and 2b.¹⁰² Subsequent synthetic activators overcame issues directly related to the lipidic structure, such as metabolic instability, poor cellular permeability, and low water solubility. Ligand-based drug design efforts led to 5, the first synthetic activator yielding cellular effects at mid-μM concentrations.¹²⁴,¹²⁵ This discovery paved the way for the development of further activators, such as 7a.¹²⁸ Although the binding mode of the analogue 7b raised some discussion¹³⁰,¹³¹ it is possible that both proposed models are valid in different conditions, and this controversy reminds us that ligand–protein interactions cannot be always recapitulated by a single-crystal structure. In any case, both 7a and its derivative 7c¹³² inhibited tumor growth in xenograft models, showing SIRT6 activation efficacy in vivo for the first time.¹²⁹,¹³² The recently described activator 10b,¹³⁸ developed using the 5 binding mode as a model, displayed efficacy at low micromolar concentrations, being an activator of both deacetylation and demyristoylation activities. Remarkably, it also possessed antitumor activity in vivo, even though it showed poor water solubility and very low bioavailability. Notably, CETSA measurements demonstrated that 10b binds to SIRT6 in cells. Nonetheless, further studies are necessary to verify whether the observed phenotypic effects are genuinely related only to SIRT6 activation. Anyhow, 10b represents a good lead compound that still necessitates a full validation and optimization of the pharmacodynamic and pharmacokinetic properties to be considered as a therapeutic option in the PDAC context.

In the case of inhibitors, the development of substrate-based peptidomimetics led to compounds 15a–f that displayed SIRT6 inhibition in the nanomolar range, although the only compound tested in vivo (15f) did not show any effect. Nonetheless, given the high potency, these compounds represent optimal starting scaffolds for further developments, first aimed at improving the cellular permeability. Differently, compounds 19b, 20b, and 21b, developed following structure- and ligand-based drug design strategies, were cellually active, although they inhibited SIRT6 enzymatic activity only in the micromolar range. 19b also displayed efficacy in a mouse xenograft model of DLBCL; however, additional analyses are necessary to demonstrate a causal correlation between its anticancer activity and SIRT6 inhibition. Therefore, currently, 19b can be considered only a hit molecule that needs complete validation and, in case, extensive optimization of potency and selectivity.

Innovative approaches relying on high-throughput compound testing hold great potential for drug discovery. Compound 22a has been discovered by means of DNA-encoded libraries,¹⁶⁹ a combinatorial approach in which the structure of each molecule is encoded by a conjugated DNA identifier sequence.¹⁷²,¹⁷³ This method allows quick testing of millions of combinations of fragments using micrograms of protein, offering the exploration of a vast chemical space. The successful application of this approach to SIRT6 led to a low micromolar inhibitor (22a) endowed with cellular activity. The application of this technique also to SIRT6 activator discovery would be very interesting.

Finally, compound 23 represents an exciting prospect. It showed low micromolar activity in vitro and was able to cause blood glucose reduction in a mouse model of diabetes.⁷¹ Moreover, its simple structure is amenable of modifications that may lead to more potent derivatives upon a proper structural optimization.

Different challenges have been characterizing the path to the discovery of SIRT6 modulators. These include initial difficulties of properly separating activation and inhibition and the suboptimal efficacy of currently discovered modulators, as explained by the absence of nanomolar activators thus far. The recent discoveries of in vivo active compounds (particularly 7c and 10b) bring good hopes for the development of further potent and selective SIRT6 activators. In the case of inhibitors, researchers managed to obtain nanomolar or low micromolar compounds such as 15f, 22a, and 23, plus the mid micromolar inhibitor 19b, which was active both in cell and in vivo, although its SIRT6 target engagement needs to be demonstrated. These molecules cover different chemical classes, ranging from peptidomimetics to small molecules, and some of them represent ideal hit/lead compounds for further development.

Anyway, additional efforts are necessary to improve the potency of the currently available SIRT6 modulators, since
usually only nanomolar compounds have concrete chances to progress to preclinical and clinical phases. To this end, structure-based drug design approaches might be particularly beneficial. Indeed, the availability of the crystal structures of SIRT6 in complex with both activators and inhibitors of polyphenolic nature, such as 3b, 3e, and 17a, enable the identification of key features for target recognition and activity modulation. These cocrysals could be exploited to develop new activating or inhibiting compounds whereby only the important hydroxyl groups are kept while removing the nonessential ones, thus abolishing their pleiotropic effects and increasing their specificity. Moreover, in order to increase potency and selectivity, scaffold hopping approaches could be applied to develop molecules bearing different core chemotypes but retaining the key moieties for SIRT6 interaction. The inspection of the X-ray solved crystal structures of SIRT6 in complex with different modulators that bind in the same pocket with similar binding modes such as the activators 3b (Figure 6B) and 5 (Figure 7A) could be also leveraged for the structural optimization by combining crucial chemical features. For instance, compound 5 might be modified through the addition of polar groups to the pyridine moiety to form hydrogen bonds with the conserved water molecules bridging to Val115 and Asp116. Moreover, the addition of large hydrophobic groups to the benzene ring could allow further hydrophobic interactions to be established with the pocket formed by Ile61, Phe82 and Phe86.

In conclusion, the available cocystal structures, along with cutting-edge approaches such as artificial-intelligence-driven drug design and DNA-encoded libraries, have great potential in allowing the evaluation of a more diverse chemical space to obtain molecules possessing drug-like properties to facilitate the discovery of new SIRT6 modulators. To date, the ideal scenario for the initial evaluation of SIRT6 targeting molecules relies on the integration of structural approaches with classical biophysical assays and modern, label-free methods such as those based on mass spectrometry, to allow reliable assessment of protein–ligand interactions and avoid false positives and negatives that may impair the following steps of a drug discovery campaign.

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**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

**Biographies**

Francesco Fiorentino graduated in Medicinal Chemistry at the University of Rome “La Sapienza” (Italy) in 1984. He received his Ph.D. in Biophysical Chemistry at University of Oxford (UK) in 2020 under the supervision of Prof. Dame Carol Robinson, working on the elucidation of the structure and regulation of membrane proteins using mass spectrometry. He is now a Postdoctoral Research Associate at the University of Oxford. His research activity has been directed toward the investigation of the molecular mechanisms underpinning protein function and modulation. To this end, he is applying native mass spectrometry and other biophysical techniques to investigate the protein complexes involved in bacterial membrane biogenesis and epigenetics.

Antonello Mai graduated in Pharmacy at the University of Rome “La Sapienza”, Italy, in 1984. He received his Ph.D. in 1992 in Pharmaceutical Sciences, with a thesis entitled “Researches on New Polycyclic Benzodiazepines Active on Central Nervous System”, with advisor Prof. M. Artico. In 1998, he was appointed Associate Professor of Medicinal Chemistry at the same University. In 2011, Prof. Mai was appointed Full Professor of Medicinal Chemistry at the Faculty of Pharmacy and Medicine, Sapienza University of Rome. He has published more than 250 papers in peer-reviewed high-impact factor journals. His research interests include the synthesis and biological evaluation of new bioactive small-molecule compounds, in particular modulators of epigenetic targets. In addition, he is working in the field of antibacterial/antimycobacterial, antiviral, and CNS agents.

Dante Rotili graduated in Medicinal Chemistry at the University of Rome “La Sapienza” (Italy) in 2003. He received his Ph.D. in Pharmaceutical Sciences at the same University in 2007. In 2009/2010, he was a research associate at the Department of Chemistry of the University of Oxford, where he worked in collaboration with Prof. C. Schofield in the development of chemoproteomic probes for the characterization of 2-oxoglutarate-dependent enzymes. In 2020, he was appointed as Associate Professor of Medicinal Chemistry at the University of Rome “La Sapienza”. Since 2017, he has had the Italian National Habilitation to Full Professor of Medicinal Chemistry. His research activity has been focusing mainly on the development of modulators of epigenetic enzymes with potential applications in cancer, neurodegenerative, metabolic, and infectious diseases.

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**ABBREVIATIONS USED**

SAC, 5-azacytidine; 5-hmC, 5-hydroxymethylcytosine; 5-mC, 5-methylcytosine; ABPP, affinity-based protein profiling; AMPK, AMP-activated protein kinase; Bax, Bcl-2-associated X protein; BER, base excision repair; CETSA, cellular thermal shift assay; CREB, CAMP response element-binding protein; CRC, colorectal cancer; DAC, decitabine; DDR, DNA-damage repair; DLBCL, diffuse large B-cell lymphoma; DNMT1, DNA methyltransferase 1; DSb, double-strand break; EMT, epithelial–mesenchymal transition; E2F1, E2 transcription factor 1;
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