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The role of structural biology in the design of sirtuin activators

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Abstract

Sirtuins are NAD^+-dependent protein lysine deacylases and mono-ADP-ribosylases whose activity regulates different pathways, including DNA damage repair, cell survival and metabolism, reactive oxygen species (ROS) detoxification, inflammation, cardiac function, and neuronal signaling. Considering the beneficial effects of specific sirtuin isoforms on health and lifespan, the past two decades have seen a mounting interest in the development of sirtuin activators. The availability of enzyme-activator co-crystal structures has proven significant throughout the years for elucidating the mechanisms of action of activators and designing more potent and selective molecules. In this review, we highlight the most interesting examples of sirtuin activators and provide comprehensive coverage of the role that structural biology played in their discovery and characterization.

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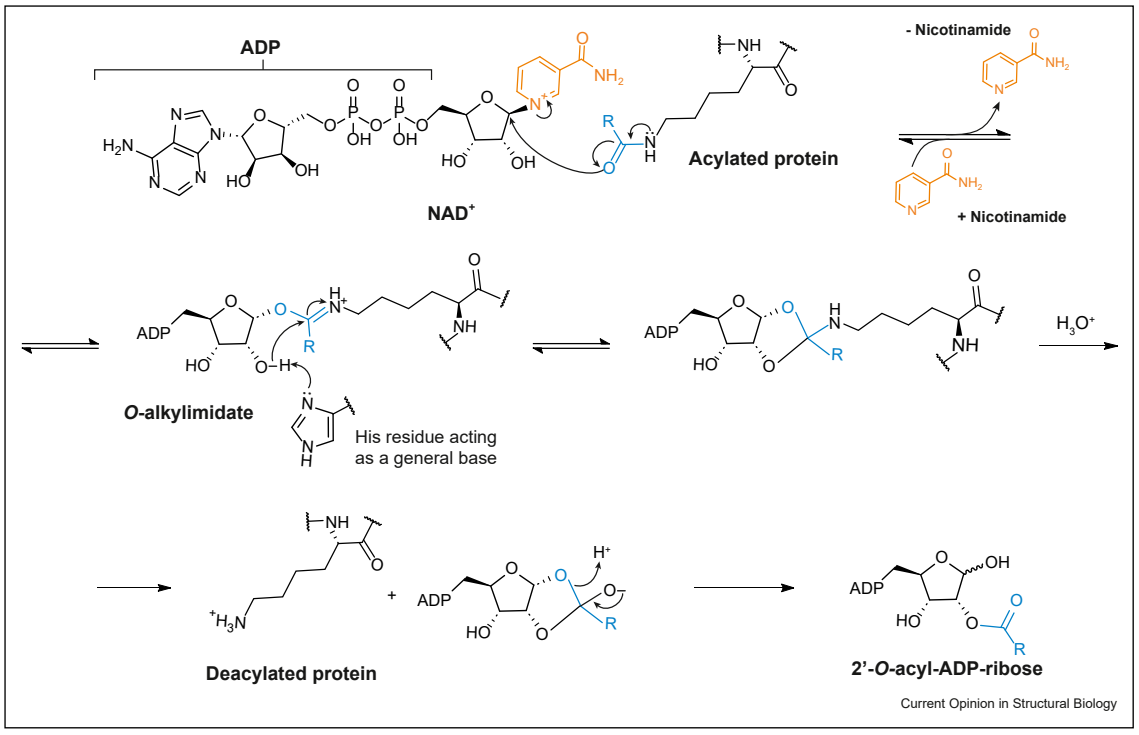
Introduction

Sirtuins are a group of different enzymes that catalyze protein lysine deacylation and mono-ADP-ribosylation through a nicotinamide adenine dinucleotide (NAD^+)-dependent catalytic mechanism. To date, seven sirtuin isoforms (SIRT1 to SIRT7) have been identified in humans, all of which share an evolutionarily conserved catalytic region of ~275 residues, while differing in the size and sequence of their *N*- and *C*-terminal domains, which affect their substrate preference, the catalyzed reaction, and subcellular localization [1,2]. The most common reaction catalyzed by human sirtuins is deacetylation, mostly mediated by SIRT1-3, with SIRT4-7 also possessing weak activity. Nonetheless, sirtuin family members also catalyze the removal of 3-hydroxybutyryl groups (SIRT3), 3-hydroxy-3-methylglutaryl (HMG) chains (SIRT4), negatively-charged acyl moieties (SIRT5 and SIRT7), and long-chain fatty acyl chains (SIRT2 and SIRT6, although *in vitro* activity has been shown also for SIRT1, 3, and 7) [1,3–11]. Furthermore, SIRT4 and SIRT6 are also endowed with a mono-ADP-ribosylase activity [12,13].

All sirtuins follow an analogous deacylation catalytic mechanism. Upon interaction of the acylated substrate with the enzyme, the carbonyl oxygen of the acyl group performs a nucleophilic attack on the NAD^+ ribose at C1', thus displacing the nicotinamide and forming an *O*-alkylimidate intermediate (Figure 1). This is followed by histidine-mediated deprotonation of the hydroxyl group at C2', which attacks the carbon of the newly-formed iminium ion, yielding a cyclic intermediate comprising C1' and C2', which is hydrolyzed, thus affording the deacylated protein and 2'-*O*-acyl-ADP-ribose as products (Figure 1) [14]. Sirtuin catalytic activity is regulated by a variety of processes, including protein–protein interactions, post-translational modifications, and interactions with endogenous molecules. Furthermore, their physiological activity is controlled both transcriptionally and through the regulation of their degradation [10].

Given the many reactions catalyzed by sirtuins, it comes as no surprise that they have pivotal functions in different cellular processes such as DNA damage repair, cell survival, metabolism, management of reactive oxygen species (ROS), inflammation, neuronal signaling, and cardiac function [15]. Several studies have shown that calorie restriction (CR) and exercise enhance the expression of SIRT1, 3, and 6, and they have been identified as significant mediators of the health benefits of CR and physical activity [16–22]. Consistent with this, their overexpression in mouse models has been shown to resemble the effects of CR

Figure 1



Schematic representing the mechanism of the protein deacylation reaction catalyzed by sirtuins.

and exercise, along with increasing their lifespan and exerting a protective role in the context of diseases such as cancer, cardiovascular disorders, and type 2 diabetes [23–32]. Similarly, SIRT5 has been indicated to exert protective roles at both neuronal and cardiac levels [33,34].

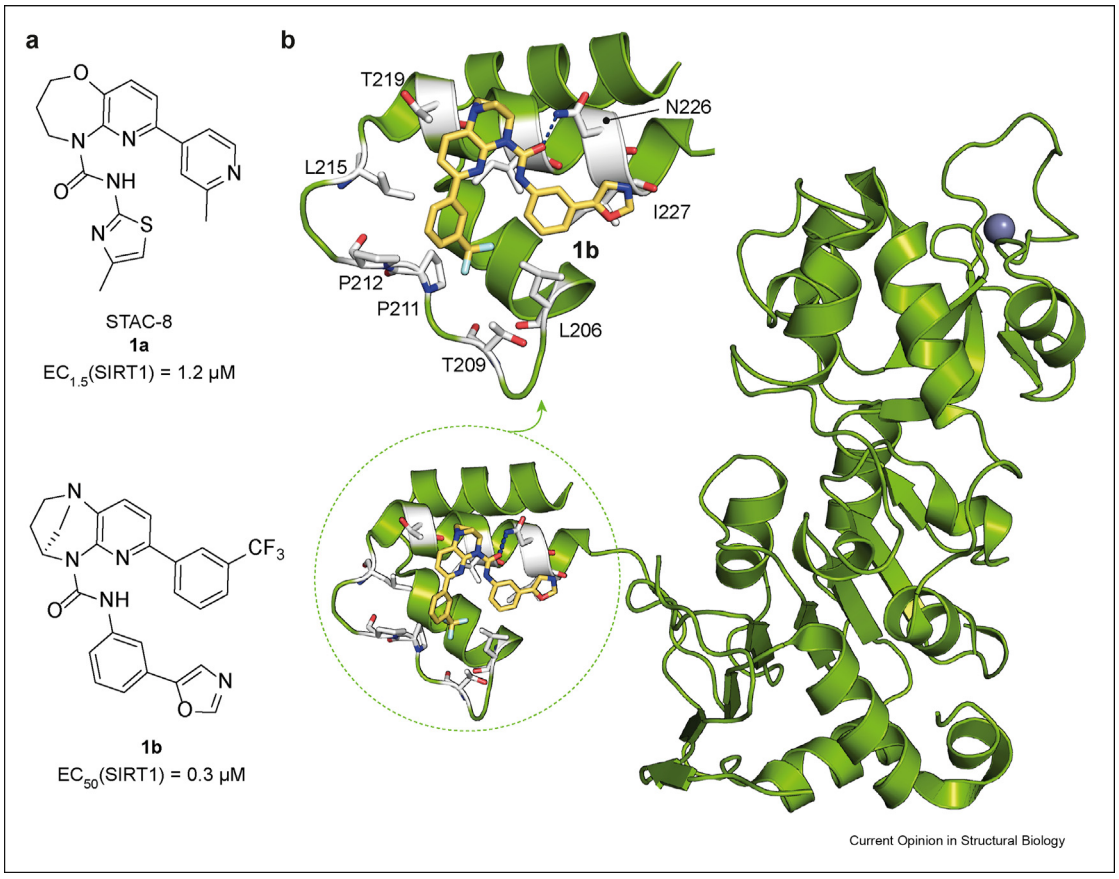
Overall, these findings are in line with the manifold roles of sirtuins in maintaining cellular homeostasis, which include the stimulation of oxidative metabolism and mitochondrial function and the control of ROS formation [35]. It should be noted that the contribution of sirtuins to carcinogenesis is highly context-dependent, as they may act as either tumor promoters or suppressors depending on the tissue type and cancer stage [35–37] and they may also contribute to the onset of other diseases such as neurodegenerative disorders. Hence, sirtuin inhibitors have been developed [35,38]. Nevertheless, given the health benefits connected to increased sirtuin activity in multiple settings (e.g., diabetes, neurological, and cardiovascular disorders), there has been increasing interest in the development of activators or enhancers of sirtuin activity [38].

In these regards, structural biology has played a key role in the design and optimization of active compounds and in the clarification of their mode of action. In this review, we discuss recent advances in the drug discovery of sirtuin activators with a specific focus on structure-based medicinal chemistry campaigns.

SIRT1 activation

From a historical point of view, it is worth mentioning that X-ray crystallography, coupled to enzymological and biophysical assays, has been crucial for the clarification of the mode of action of the so-called SirTuin-Activating Compounds (STACs). This term refers to a chemically heterogeneous class of molecules that have been shown to enhance SIRT1 activity. Following the reports indicating that resveratrol could act as an allosteric activator of SIRT1 [39], medicinal chemistry efforts led to the development of synthetic STACs, which include imidazothiazole derivatives (e.g., SRT1460, SRT1720, SRT2104, and SRT2183), the imidazopyridine STAC-5, and urea-based compounds such as STAC-8 (**1a**) (Figure 2a), some of which have entered clinical trials for various therapeutic indications [40,41]. Nevertheless, concerns have been raised over the years as to whether these compounds actually bind and activate SIRT1, or if the reported effects were an artefact of bioactivity screens utilizing non-natural peptide substrates with large hydrophobic fluorescent moieties [42–44]. Nonetheless, **1a**, was shown to increase SIRT1 activity by approximately 50% ($EC_{1.5}$) at 1.2 μM (Figure 2a) in a mass spectrometry (MS)-based assay

Figure 2



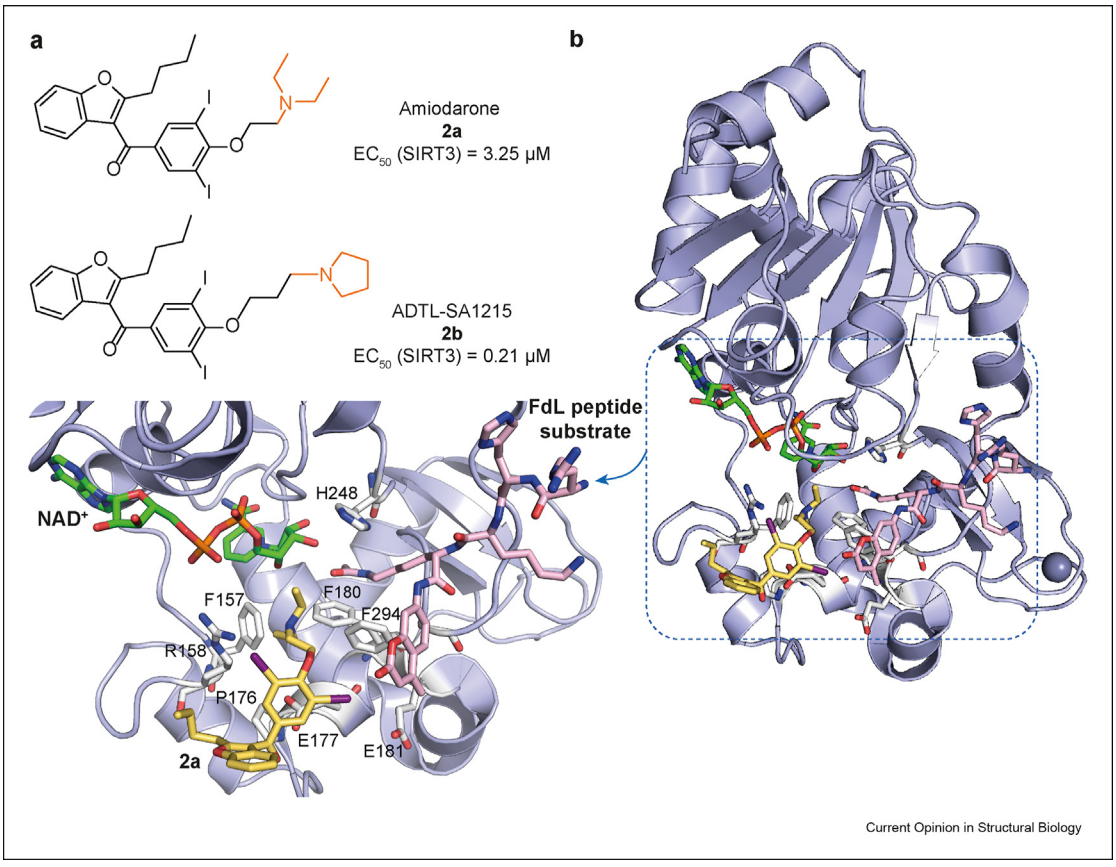
(**a**) Structures and activity data of SIRT1 activators **1a, b**. (**b**) Co-crystal structure view of mini-hSIRT1 in complex with **1b** (PDB ID: 4ZZH) indicating the main residues involved in the interaction between the activator and the enzyme. Mini-hSIRT1 is colored in green (key residues are in white); **1b** is depicted as yellow sticks; hydrogen bonds are shown as blue dashed lines.

monitoring the SIRT1 product *O*-acetyl adenosine diphosphate ribose (OAcADPR), rather than a fluorescence-based assay. Moreover, an X-ray co-crystal structure of an analogue of **1a**, compound **1b** [half-maximal effective concentration (EC_{50}) = 0.3 μM in the OAcADPR MS-based assay] (Figure 2a), with an engineered human SIRT1 (mini-hSIRT1), combined with hydrogen–deuterium exchange mass spectrometry (HDX-MS) demonstrated that STACs do bind with SIRT1 and allosterically activate it by interacting with a shallow hydrophobic region at SIRT1 *N*-terminus (Figure 2b), which was called STAC-binding domain (SBD) [45]. These findings support the observed structure-activity relationships (SAR) for STACs, which indicated the need of a planar scaffold to efficiently activate SIRT1 [46]. Biochemical studies also indicate that fluorescent moieties in the substrate peptide are not necessary for SIRT1 activation [47], but rather bulky hydrophobic amino acids (Trp, Tyr or Phe) at the +1 and +6 positions relative to the acetyl-Lys are required for activation by STACs [39,41,48]. HDX-MS experiments indicate that binding of **1b** to SIRT1 causes a conformational change that determines an increase of substrate binding, thereby increasing SIRT1 catalytic efficiency [45]. Moreover, Glu230 was shown to be essential for SIRT1 activation by **1a** and **1b** [41,45]. Although not directly involved in activator binding, Glu230 stabilizes the activated conformation of SIRT1 by engaging in an electrostatic interaction with Arg446, which is located in the catalytic site upon STAC binding [45].

SIRT3 activation

SIRT3 activation is a relatively new topic, with few studies describing SIRT3 activators being published in the past few years [49,50]. Recently, a structure-guided medicinal chemistry campaign led to the development of a 2-butylbenzofuran-derivative acting as SIRT3 activator and which was shown to modulate autophagy in triple negative breast cancer (TNBC) cells [50]. The study started with docking experiments that identified amiodarone (**2a**), a known antiarrhythmic drug [51] (Figure 3a), as the lead molecule that activated SIRT3 with an EC_{50} of 3.25 μM in a Fluor de Lys (FdL) assay, which employs a peptide substrate carrying the 7-

Figure 3



(**a**) Structures and activity data of SIRT3 activators **2a, b**. (**b**) Co-crystal structure view of SIRT3 in complex with **2a** and the FdL peptide substrate (PDB ID: 5H4D) indicating the main residues involved in the interaction between the activator and the enzyme. SIRT3 is colored in light blue (key residues are in white), **2a** is depicted as yellow sticks, the FdL peptide substrate is shown as pink sticks.

amino-4-methylcoumarin (AMC) fluorophore. An X-ray co-crystal structure of the SIRT3/NAD^+/FdL peptide substrate/**2a** complex revealed that **2a** interacts with SIRT3 at the entry of its acyl-binding channel (Figure 3b). Comparison with the structure of SIRT3 in the absence of the activator, suggested that, upon **2a** binding, SIRT3 undergoes a conformational change that brings Phe157 closer to NAD^+, thus enhancing the π-stacking with the nicotinamide moiety. Hence, the increased interactions between Phe157 and nicotinamide may facilitate the dissociation of nicotinamide, thus promoting the deacetylation reaction. Similarly, upon **2a** binding, the side-chain of His248 becomes closer to the ribose moiety, a movement which was suggested to promote the removal of the acetyl group. Altogether, the conformational changes involving Phe157 and His248 are pivotal for SIRT3 activation. In addition, the phenyl ring of **2a** forms a π-anion interaction with Glu177, while the nitrogen of the diethylamine moiety was not at the right distance to form a salt bridge with Glu177 (Figure 3b). Nonetheless, the *N*-ethyl groups of **2a** were shown to form hydrophobic interactions with Phe157, Pro176, Ile179, Phe180, and Phe294 and thus contribute to SIRT3 activation. Based on these findings, the authors designed derivatives bearing different chains in place of the diethylamine moiety, in order to maximize the interactions with the nearby residues. This investigation led to ADTL-SA1215 (**2b**) which is characterized by the presence of a propyl spacer rather than an ethyl one and by the conformational restriction of the ethyl groups inside a pyrrolidine ring. These modifications led to a 15-fold increase in potency [EC_{50}(**2b**) = 0.21 μM in the same FdL assay], with **2b** also shown to be selective at 100 μM over SIRT1, 2, and 5. Notably, **2b** was shown to reduce the acetylation levels of SIRT3 substrate SOD2 in MDA-MB-231 TNBC cells and cellular thermal shift assays (CETSA) demonstrated that compound **2b** increases the thermal stability of SIRT3, indicating that it binds directly to SIRT3, whereas it had little effect on the thermal stability of SIRT1, SIRT2, and SIRT5, indicating that it does not interact with these sirtuin isoforms. Compound **2b** also exhibited low-micromolar antiproliferative activity in MDA-MB-231 cells, along with inhibition of autophagy and mitophagy, and could also reduce tumor size in an MDA-MB-231 xenograft

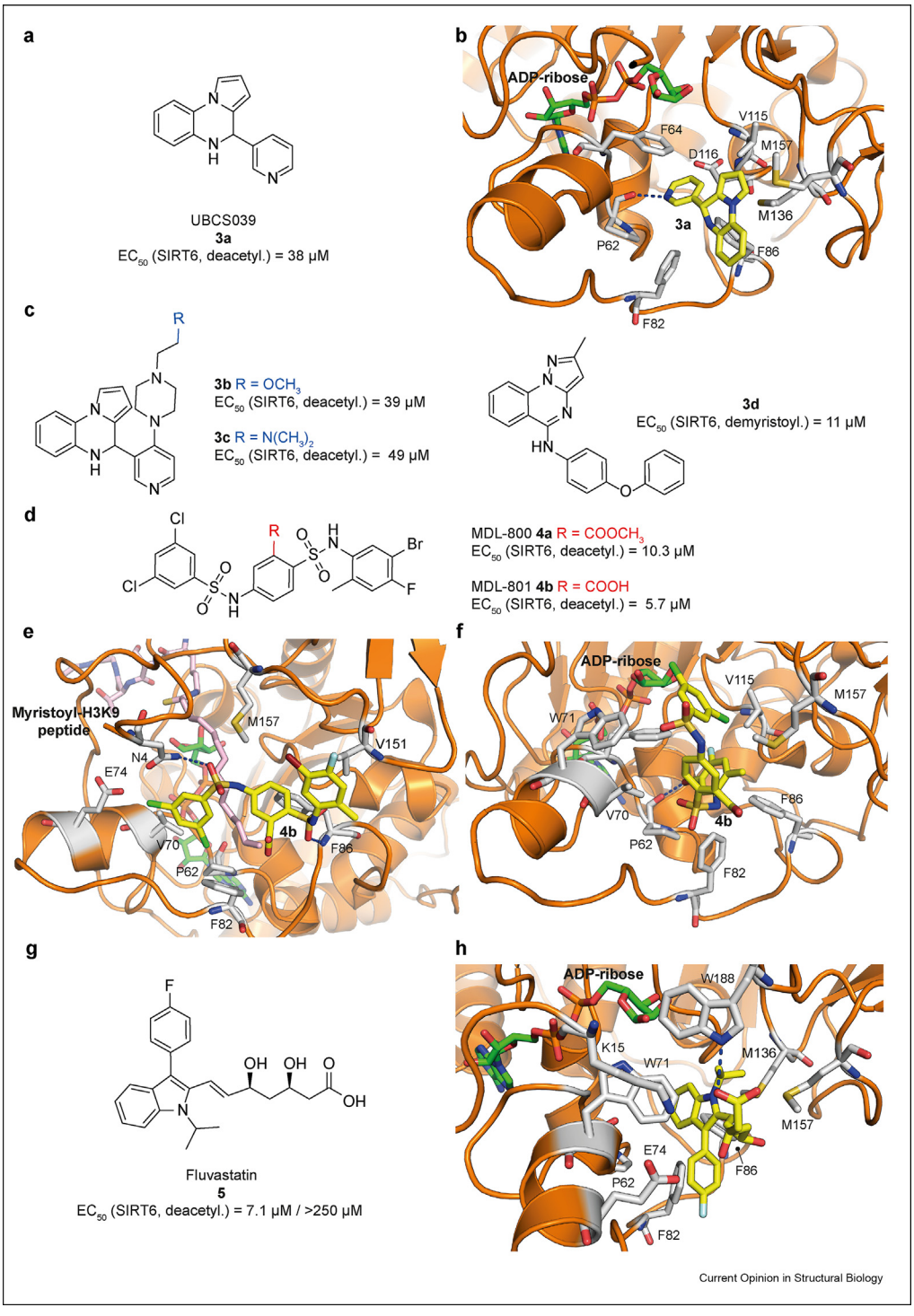
mouse model. Docking experiments indicated that the binding mode of **2b** was nearly identical to that of **2a**, and in this case the pyrrolidine moiety was able to form a salt bridge with Glu177 and was inserted deeper into the allosteric pocket [51]. These features may account for the increased potency of **2b**, although a co-crystal structure is necessary to confirm the computational data.

SIRT6 activation

Early studies showing that free fatty acids can enhance the deacetylase activity of SIRT6 [52,53] laid groundwork for the development of SIRT6 activators. The first synthetic SIRT6 activator is UBCS039 [**3a**, EC_{50}(deacetylation) = 38 μM, 3.5 max activation in a peptide deacylation assay monitoring nicotinamide production] (Figure 4a) [54,55], the co-crystal structure of which in complex with SIRT6 and ADP-ribose provided a structural basis for activator design. Specifically, **3a** was shown to bind to the hydrophobic acyl-binding channel situated at the end of the catalytic region, and forms a key hydrogen bond with Pro62, a residue of crucial importance for SIRT6 modulators binding (Figure 4b) [54]. These findings uncovered a SIRT6-specific binding site that might be exploited for activation and stimulated further research which led to the development of a series of **3a** derivatives bearing the same pyrrolo[1,2-*a*]quinoxaline core. Among them, **3b** and **3c**, possessing a piperazinyl moiety at C4 position of the pyridine ring, exhibited the highest activities in a SIRT6 fluorescence-based FdL assay (Figure 4c) [56]. Compound **3b** was also shown to enhance SIRT6-mediated H3K9 deacetylation in non-small cell lung cancer (NSCLC) H1299 and hepatocellular carcinoma (HCC) PLC/PRF/5 cells [56]. Docking analysis implies that **3c** binds in the same pocket as **3a**, but in a different conformation, with the pyridine ring no longer pointing towards Pro62, while the protonated nitrogen of the dimethylamino group on the side chain forms π-cation interactions with Trp188 [56]. Another group has developed a series of pyrazolo[1,5-*a*]quinazoline derivatives guided by docking analysis based on the SIRT6-**3a** co-crystal structure, which finally led to compound **3d** (Figure 4c), which was shown to enhance SIRT6-mediated demyristoylation with an EC_{50} value of 11 μM in the FdL assay [57]. Moreover, CETSA performed in mouse embryonic fibroblasts (MEFs) showed that **3d** could stabilize SIRT6, thereby indicating cellular target engagement [57].

A different approach has been exploited by Huang and colleagues, who developed the bis-benzenesulfonamide derivative MDL-800 (**4a**) and its corresponding carboxylic acid MDL-801 (**4b**), which activated SIRT6-mediated deacetylation with EC_{50} values of 10.3 and 5.7 μM in FdL assays (Figure 4d), respectively, while being selective over HDAC1-11 and SIRT1-5, and 7. Notably, the cell-permeable **4a** was shown to dose-dependently decrease the acetylation levels of H3K9 and H3K56 in HCC cell lines and impair HCC proliferation both in cells and *in vivo* [58]. Interestingly, a co-crystal structure of SIRT6 bound to **4b**, H3K9 myristoylated peptide, and ADP-ribose revealed that the compound binds to a surface site distinct from that of 3, located in a distal portion of the acyl-binding channel. This region is defined by the first seven residues of the SIRT6 *N*-terminus Val70, Glu74, Phe82, Phe86, Val153, and Met157 (Figure 4d). The dichlorobenzene portion of **4b** fits within a space defined by Asn4, Val70, and Glu74 and forms weak polar interactions along with π-π interactions with Phe82. In addition, the sulfonamide attached to the chlorobenzene forms a hydrogen bond with Asn4. The central benzene ring forms a T-shaped π-stacking interaction with the side chain of Phe86, while its backbone amide forms a hydrogen bond with the activator sulfonamide. The necessity of the π-stacking in which Phe86 is involved was demonstrated through its mutation into an Ala, which leads to reduced potency of both **4a** and **4b**. Finally, the 5-bromo-4-fluoro-2-methylaniline moiety seems solvent-exposed (Figure 4e) [58]. Recently, this binding mode was challenged by You and Steegborn, who reasoned that the electron density assigned to **4b** was attributable to morpholinoethanesulfonic acid (MES) present in the crystallization buffer [59]. To support this argument, they co-crystallized both $SIRT6_{3-308}$ and its *N*-terminal truncated version $SIRT6_{13-308}$ with ADP-ribose either in the presence or absence of **4b**. According to their X-ray crystallography data, **4b** binds in the same region as compound 3, rather than in the distal site of the acyl-binding channel proposed by Huang et al. In this structure **4b** exhibits an opposite orientation as compared to the previously reported one, with the dichlorobenzene ring being solvent exposed and the 5-bromo-4-fluoro-2-methylaniline moiety positioned deep within the binding pocket. Specifically, this portion enters a hydrophobic cleft defined by Phe64, Val 70, Phe82, Phe86, and Val115, with the bromine atom forming a halogen bond with the oxygen of Pro62 backbone amide (Figure 4f). The central benzene ring of the activator is now sandwiched between Met157 and a hydrophobic region formed by Val70 and Trp71. More recently, Huang et al. crystallized SIRT6 again under the same conditions as their original study [60]. The new X-ray crystal structure obtained without **4b** demonstrated that MES does not fit correctly in its initially proposed putative binding site. Moreover, a new structure solved in the presence of **4b**, albeit having a lower overall resolution (3.2 Å vs 2.5 Å), exhibits improved electron density for the activator and validates their earlier results [60]. It is worth noticing that the co-crystal structures solved by Huang and colleagues include H3K9 myristoylated peptide, which is absent in those obtained by You and Steegborn. Hence, the variations in the binding mode of **4b** can be attributed to simultaneous peptide substrate binding, which

Figure 4



(**a**) Structure and activity data of SIRT6 activators UBCS039 (**3a**). (**b**) Co-crystal view structure of SIRT6 in complex with **3a** and ADP-ribose (PDB ID: 5MF6) indicating the main residues involved in the interaction between the small molecule and the enzyme. (**c**) Structures and activity data of SIRT6 activators **3b-d**. (**d**) Structures and activity data of SIRT6 activators **4a, b**. (**e**) Co-crystal structure reported by Huang and colleagues of SIRT6 in complex with **4b**, ADP-ribose, and myristoylated H3K9 peptide (PDB ID: 5Y2F) indicating the main residues involved in the interaction between the small molecule and the enzyme. (**f**) Co-crystal structure reported by You and Steegborn of SIRT6 in complex with **4b** and ADP-ribose (PDB ID: 6XVG) indicating the main residues involved in the interaction between the small molecule and the enzyme. (**g**) Structure and activity data of SIRT6 activator fluvastatin (**5**). (**h**) Co-crystal structure of SIRT6 in complex with **5** and ADP-ribose (PDB ID: 6ZU4) indicating the main residues involved in the interaction between the small molecule and the enzyme. SIRT6 is colored in orange (key residues are in white), compounds are depicted as yellow sticks, ADP-ribose is shown as green sticks, myristoylated H3K9 peptide is shown as pink stick, hydrogen bonds are shown as blue dashed lines.

impacts on the small molecule interactions with SIRT6. Since crystal structures are only snapshots of a protein's conformational state, which can be altered upon interaction with other ligands or substrates, it is plausible that the structures solved by the two groups reflect two distinct protein states governed by the presence of the substrate. Nevertheless, more investigations, such as the X-ray crystal structure of **4b** in the presence of an acetylated substrate, might assist in settling this debate.

The HMG-CoA reductase inhibitor fluvastatin (**5**, Figure 4g), which is used as a therapeutic for hypercholesterolemia, has been identified as a SIRT6 activator with an EC_{50} value for of 7.1 μM in a SIRT6 deacetylase assay employing a fluorophore-containing peptide substrate [61]. Consistent with this, **5** was shown decrease the acetylation levels of H3K9 and H3K56 in HCC HepG2 cells. Recently developed FdL and MS-based assays have, however, implied a substantially greater EC_{50} (>250 μM) for 5, with a maximum activation of 3.5-fold at 1 mM [62]. Nevertheless, You and Steegborn recently revealed a co-crystal structure of $SIRT6_{13-308}$ bound to **5**, which indicated that **5** binds at the exit of the acyl-binding channel [62]. The heptanoic acid chain fits inside a pocket formed by Lys15, Trp71, and Glu74 with carboxylic acid of **5** functioning as an anchoring point by forming a hydrogen bond with Trp188. The indole portion of **5** is directed towards the hydrophobic region established by Phe64, Phe82, Phe86, Ile61, Pro62, and Met136 (Figure 4h). However, bulky substituents, such as the isopropyl and fluorophenyl groups, which form only weak hydrophobic interactions with surrounding residues (Met157 for the isopropyl moiety, Val70 and Pro80 for the fluorophenyl ring), hinder a deeper entrance of **5** into the pocket and cause it to assume a conformation that is incompatible with establishing important polar interactions with the backbone oxygen of Pro62 [62]. Thus, X-ray crystallography was essential to rationalize the weak potency of compound **5** and provided a structural basis for the design of new activators possessing the same core structural scaffold but having alternative substituents.

Conclusions

With the discovery of resveratrol as the first small molecule sirtuin activator, research into sirtuin activation has proliferated. In parallel with this, structural biology has enabled for the full characterization of the structure of all sirtuin isoforms and the explanation of the binding mode of various activators, thereby uncovering yet unknown allosteric sites. In this review, we have outlined recent advances in structure-guided sirtuin activator drug development, including the co-crystal structures of compounds bound to SIRT1, SIRT3, and SIRT6.

In this context, X-ray crystallography has played a key role and provided a significant contribution to the field by elucidating the binding mode of STACs, whose mechanism of SIRT1 activation had been questioned for a long time [45]. The discovery that the antiarrhythmic drug amiodarone (**2a**) enhances SIRT3 activity has laid groundwork for the development of selective SIRT3 activators. The SIRT3-**2a** co-crystal structure was crucial in the development of the derivative **2b**, which exhibits a 15-fold increase in potency [51]. A controversy has also arisen over the binding mode with SIRT6 of **4b**, with two separate groups reporting substantial discrepancies in its key interactions. X-ray crystallography has been central in defining the key interactions and possible modification sites of fluvastatin (**5**) as a SIRT6 activator [62].

In the next few years, the integration of structural biology with biochemical assays and innovative biophysical methods [63,64] will further facilitate the development of new sirtuin activators. To this end, the above-mentioned HDX-MS along with native mass spectrometry represent ideal orthogonal techniques to evaluate the activation mechanism of sirtuin activators [64,65]. Moreover, in circumstances where X-ray crystallography may not enable the resolution of protein or protein-ligand complex structures, advances in cryo-electron microscopy (cryo-EM) [66,67] will be essential in bridging this gap and might expedite drug development programs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Abbreviations

AMC 7-Amino-4-methylcoumarin
CETSA cellular thermal shift assay
CR calorie restriction
$EC_{1.5}$ activity increase by 50%
EC_{50} half-maximal effective concentration
FdL Fluor de Lys
HDX-MS hydrogen−deuterium exchange mass spectrometry
HCC hepatocellular carcinoma
HMG 3-hydroxy-3-methylglutaryl
MEF mouse embryonic fibroblast
MES morpholinoethanesulfonic acid

MS mass spectrometry
NAD^+ nicotinamide adenine dinucleotide
NSCLC non-small cell lung cancer
OAcADPR *O*-acetyl adenosine diphosphate ribose
ROS reactive oxygen species
SAR structure–activity relationships
STAC sirtuin-activating compound
TNBC triple negative breast cancer

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* of special interest
** of outstanding interest

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