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Activators of Sirtuin-1 and their Involvement in Cardioprotection

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Abstract: SIRT1 is a nicotinamide adenosine dinucleotide (NAD^+)-dependent deacetylase, which removes acetyl groups from many target proteins, such as histone proteins, transcription factors and cofactors. SIRT1-catalyzed deacetylation of these factors modulates the activity of downstream proteins, thus influencing many biological processes. SIRT1 is involved in the regulation of metabolism, inflammation, and tumor growth. The activity of this enzyme is related to the beneficial health effects of calorie restriction, such as lifespan extension and, in particular, the activation of SIRT1 has a positive impact on the cardiovascular system. Therefore, SIRT1 is considered as an attractive drug target and modulation of SIRT1 may represents a new therapeutic strategy against cardiovascular diseases, as small molecules able to activate SIRT1 can be considered as cardioprotective agents. In this review, we summarize both natural or synthetic compounds developed as SIRT1 activators, with a focus on their promising therapeutic applications in cardiovascular pathologies.

Keywords: SIRT1, activators, STACs, cardioprotection, NAD, deacetylation.

1. INTRODUCTION

Silent information regulator 2 (Sir2) is a protein family of enzymes that is ubiquitous, being present in both prokaryotes and eukaryotes. After the discovery of the yeast Sir2 protein, the mammalian homologues of Sir2, sirtuins, were identified and studied [1]. The sirtuin family comprises seven NAD^+-dependent enzymes (from SIRT1 to SIRT7) belonging to class III human histone deacetylases (HDACs). They differ for subcellular localization, tissue specificity and targets. In particular, Sirtuin-1 (SIRT1), the most extensively studied and characterized subtype, is predominant in the nucleus but it is also expressed in the cytoplasm under certain conditions [2]. SIRT1 is the enzyme responsible for the reversible deacetylation of ε-acetyl lysine side chains of target proteins by using NAD^+ as the cofactor, producing the deacetylated substrate, together with nicotinamide and 2'-*O*-acetyl-ADP-ribose. SIRT1 deacetylates both histones and non-histone proteins. The deacetylation reaction proceeds in two steps: 1) NAD^+ is cleaved and ADP-ribose is covalently attached to the acetyl group of the substrate; 2) hydrolysis of the acetyl-lysine bond liberates 2'-*O*-acetyl-ADP-ribose. In this context, nicotinamide behaves as a product inhibitor and high nicotinamide levels in cells can inhibit sirtuin activity. Basically, all the sirtuins share the same conserved catalytic core, whereas the *C*- and *N*-terminal portions determine the cellular localization and the activity regulation of the sirtuins. The active site is located in a cleft between two subdomains of the catalytic core, consisting of a large hydrophilic Rossmann-fold domain (typical of many NAD^+/NADH-binding enzymes) and a smaller zinc-binding domain. The *N*-terminal extension contains the allosteric site for SIRT1-activating compounds (STACs).

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SIRT1 regulates the transcription, since it catalyzes the deacetylation of histone proteins such as histone 3 acetylated on lysine 9 (H3K9Ac) and histone 4 acetylated on lysine 16 (H4K16Ac), thus coordinating the formation of heterochromatin, and also histone 1 acetylated on lysine 26 (H1K26Ac). SIRT1 deacetylases also a broad range of non-histone proteins, such as many transcription factors and cofactors. Among them, one of the most known SIRT1 target is the p53 protein, which is deacetylated at the K382 residue. Therefore, SIRT1 negatively regulates p53-mediated transcriptional activation and this regulation allows cells to resist to DNA damage and oxidative stress-induced apoptosis, leading to increased cell survival and neuroprotective effects. The decreased activity of p53 induced by SIRT1 has been associated to the development of cancer, but contrasting data about the role of SIRT1 in cancer are present in literature. Interestingly, SIRT1 plays a controversial dual role in cancer, since it deacetylates and deactivates both tumor suppressors and oncoproteins. Moreover, SIRT1 is overexpressed in some tumours and acts as a tumour promoter, but it also exerts opposite effects, since it inhibits inflammation and preserves genomic stability [3]. A further widely recognized target of SIRT1 is nuclear peroxisome proliferator-activated receptor gamma (PPARγ), whose repression provokes fat mobilization in adipocytes and regulation of gluconeogenesis and glycolysis. Deacetylation of mitochondrial peroxisome proliferator-activated receptor γ coactivator 1 alpha (PCG-1α) by SIRT1 induces an increase of both mitochondria size and number, and promotes gluconeogenesis and fatty acid oxidation. A well characterized SIRT1 effect is to suppress nuclear factor kappa B (NFκB)-dependent inflammatory responses and also to improve stress-resistance and to influence metabolism through class O forkhead box (FOXO) transcription factors. Moreover, SIRT1 overexpression is associated with lifespan extension and health effects of calorie restriction. Acetylation and deacetylation is an important mechanism to modify and regulate the activity of proteins at the post-

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transcriptional level and SIRT1 plays a key role in a wide range of pathologies and biological processes, such as obesity, diabetes, metabolism, cancer, inflammation, cardiovascular diseases and aging-related pathologies. The contribute of SIRT1 to the regulation of many physiological signaling pathways makes this protein an interesting therapeutic target.

Many different compounds belonging to several chemical classes were used as tools to investigate the activation mechanism of SIRT1, and in this review we focus our discussion on the representative STACs that are reported in scientific papers [4-6].

Among all the sirtuins, SIRT1 is the most important regulator of the cardiovascular system, since the SIRT1-regulated downstream pathways are involved in the normal physiology of heart and vessels and also in pathologies and defects of the cardiovascular system [7-9]. SIRT1 modulates the physiology of endothelial cells by promoting vasodilation and regeneration of the vessel walls. SIRT1 protects endothelial cells from premature senescence induced by oxidative stress, and promotes the differentiation of endothelial progenitor cells (circulating cells endowed of the capacity to repair and regenerate endothelial cells) into endothelial cells [10, 11]. SIRT1 protects the heart from aging, oxidative stress, ischemia/reperfusion (I/R) injury, inflammation, hypertrophy and cardiomyocytes apoptosis [12-15]. SIRT1 was effective in acute cardioprotection against I/R injury and its involvement in cardiac ischaemic preconditioning (IPC), which is a mild ischaemic stress that activates signalling pathways leading to protection against long term I/R injury, was observed [16, 17]. SIRT1 acts as an anti-atherosclerosis factor, thanks to its anti-inflammatory, anti-oxidant and anti-apoptotic effects elicited in the endothelium [18, 19]. In addition to these functions, SIRT1 modulates vascular growth by controlling the angiogenic activity of endothelial cells and prevents endothelial senescence and dysfunctions [20, 21]. The modulatory activity of SIRT1 in the cardiovascular system is carried out through a series of mediators such as: endothelial nitric oxide synthase (eNOS), responsible of NO generation that results in endothelium vasodilation; the transcription factor forkhead box protein O1 (FOXO1), which is involved in the regulation of cellular stress; NF-κB transcription factor which is inhibited by SIRT1 to reduce inflammation and progression of atherosclerosis; p53 which, after deactivation by SIRT1, reduces cardiomyocytes apoptosis; and, finally, the angiotensin II type 1 receptor (AT1R), which is inhibited by SIRT1, thus resulting in a control of the regulation of blood pressure [22].

In this review, we focus our attention on activators of human SIRT1 and their therapeutic applications, with a particular attention to their positive effects on the cardiovascular system.

2. SIRT1 ACTIVATORS AND THEIR THERAPEUTICAL PONTETIAL AS CARDIOPROTECTIVE AGENTS

2.1. *Natural activators*

Many known SIRT1 activators are natural compounds and among them the most representative chemical classes are polyphenolic compounds and flavonoids, as described below.

Resveratrol **1** (*trans*-3,5,4'-trihydroxystilbene, Figure 1) is a dietary polyphenol isolated from edible materials such as red wine, grape skins, and peanuts, and it is one of the most widely studied natural stilbenoids, since it shows remarkable cancer chemopreventive and cardioprotective activities [23-25]. Resveratrol was found to activate SIRT1 in a screening of compound libraries by an *in vitro* fluorescence deacetylation assay using *h*SIRT1: its activity on SIRT1 was expressed as ratio to control, resulting to be 13.4 ± 1.0 when tested at a concentration of 100 μM. Interestingly, it was observed that resveratrol mimicked calorie restriction and increased lifespan in *Saccharomyces cerevisiae* by 70%, directly stimulating the activity of the yeast SIRT1 homologue, Sir2 [26]. In the same screening other polyphenols in particular, the analogous stilbene derivative piceatannol **2** (Figure 1), the two chalcone derivatives butein and isoliquiritigenin (**3** and **4**, respectively, Figure 1) and the two flavone compounds fisetin and quercetin (**5** and **6**, respectively, Figure 1) were found to activate SIRT1 with a potency similar or even slightly better than resveratrol. A SAR analysis revealed that the *trans*-stilbene scaffold is superimposable to the two flavonoid rings, arranging the peripheral phenolic groups in a similar disposition. Butein and fisetin improved significantly the lifespan of *S. cerevisiae*, whereas quercetin and piceatannol did not influence lifespan. Later it was observed that resveratrol increased lifespan also of the metazoans *Caenorhabditis elegans* and *Drosophila melanogaster* in a Sir2-dependant manner [27]. Moreover, quercetin proved to increase mRNA expression of SIRT1 and of other mitochondrial biogenesis markers in an experimental mouse model, resulting in an increased maximal endurance running capacity and voluntary wheel-running activity [28]. The first promising results in yeasts encouraged the translation of the studies of resveratrol *in vivo*, to determine if resveratrol could have similar effects on mice. Resveratrol was able to shift the physiology of middle-aged mice that were fed with a high-calorie diet (60% of calories from fat) towards that typical of mice on a standard diet, without a real reduction of calorie intake, thus increasing their survival. The risk of death of the group of mice fed with high-calorie diet was reduced still maintaining a good quality of life, as demonstrated by their improved motor skills and maintenance of body weight. Resveratrol improved the sensitivity to insulin by an AMP-activated protein kinase (AMPK)-mediated mechanism and increased the number of mitochondria. However, the improvement in general health in mice was not demonstrated to be correlated with a direct action of resveratrol on SIRT1 [29]. The influence of resveratrol on AMPK activity was later confirmed, since activation of SIRT1 contributed to an increased expression of AMPK, which is a serine/threonine

kinase activated by an increase of cellular AMP-to-ATP ratio, acting as a sensor of cellular energy status. These series of events finally resulted in an improved cardiac function, therefore these implications may be useful for counteracting heart failure and other cardiac pathologies. On the contrary, a reduced cardiac function and AMPK expression were observed in SIRT1 knockout mice [30].

Many studies reported in the literature describe the activation of SIRT1 by resveratrol, focusing in particular on the beneficial cardioprotective effects elicited by resveratrol that are mediated by SIRT1 activation [31]. Resveratrol was able to reduce ROS formation in cardiomyocytes by a SIRT1-mediated mechanism [32], and to stimulate mitochondrial biogenesis in both endothelial cells and in the aortas of type 2 diabetic mice through the activation of SIRT1 and the upregulation of endothelial NO synthase (eNOS) [33]. Resveratrol improved the beneficial effects of exercise training in aging rat hearts, since activation of SIRT1 blocked the transcription factor forkhead box O3a protein (FOXO3) accumulation in the nucleus and inhibited cell death [34]. Resveratrol induced the expression of the transcription factor KLF2 in human endothelial cells through SIRT1 and this led to an endothelial vasoprotective effect [35]. Among the many signaling pathways influenced by resveratrol, SIRT1 activation ameliorated dystrophic cardiomyopathy by targeting transcriptional co-activator p300: cardiac p300 protein was deacetylated by SIRT1, thus promoting its ubiquitination and degradation [36]. The potential of resveratrol in treating cardiomyopathy in patients with muscular dystrophies was later confirmed in two different animal models of muscular dystrophies (δ-sarcoglycan-deficient TO-2 hamsters and dystrophin-deficient mdx mice), in which resveratrol prevented cardiac hypertrophy and fibrosis and preserved cardiac function, thus increasing the survival of the animals [37]. Cigarette smoking is a major risk factor for cardiovascular diseases and resveratrol exerted a protective effect in cultured coronary arterial endothelial cells against the negative influence of smoking, preventing the oxidative stress, apoptosis and the up-regulation of inflammatory markers. These effects were mediated by SIRT1, since SIRT1 overexpression mimicked the anti-inflammatory action of resveratrol [38].

Pterostilbene **7** (Figure 1) is a natural analogue of resveratrol, which displays a better pharmacokinetic profile than resveratrol itself. Pterostilbene was able to alleviate I/R injury by activating SIRT1, since pterostilbene increased both SIRT1 protein and its mRNA expression, but no evidence of a direct interaction with SIRT1, and its subsequent activation, was reported [39].

The ability of resveratrol to activate SIRT1 has been controversially debated, since it failed to stimulate deacetylation of many physiological substrates. The first reported studies about the mechanism of SIRT1 activation by resveratrol revealed that resveratrol action was highly dependent on the presence of a non-physiological fluorophore (7-amino-4-methylcoumarin, rhodamine 110 or the most known Fluor de Lys), which was covalently attached to the acetylated peptidic substrate of the enzymatic reaction (p53). Differently, resveratrol had no activity on acetylated peptides lacking the fluorophore moiety. This controversial effect was verified both in enzymatic and in cell-based assays, by using peroxisome proliferator-activated receptor γ coactivator 1 alpha (PCG-1α) protein, which is a physiological substrate of SIRT1 [40]. In both cases, resveratrol did not change the acetylation level of PCG-1α [41]. The hypothesized mechanism implied that resveratrol binding to the enzyme provoked a conformational change which allowed the fluorophore-containing substrate to bind more tightly to SIRT1, thus mimicking enzymatic activation [42, 43]. This observation could be detrimental for the potential applications of resveratrol, since the putative pharmacological effects of this natural compound needed to be revisited in the light of these new findings. Recently, a wide peptide microarray system on physiological decetylation sites revealed that resveratrol was able to affect SIRT1 activity depending on the substrate sequence. In particular, large and hydrophobic residues at several positions C-terminal to the acetyl-lysine were preferred, whereas positively charged residues hindered resveratrol effects [44]. A recent study was focused on the role of resveratrol in the binding process between SIRT1 and fluorescent peptidic substrates. In particular, the authors of this study solved the structure of SIRT1 in complex with resveratrol and the four-residue acetylated p53 peptide bearing at its C-terminal the fluorophore 7-amino-4-methylcoumarin (p53-AMC). The presence of three molecules of resveratrol was observed in the crystal structure and two of them had the function of mediating the interaction between the peptidic substrate and the N-terminal domain of SIRT1: resveratrol acted as a bridge by keeping together the N-terminal domain and the peptide, while interacting mainly with the peptidyl fluorophore and with specific residues of the N-terminal domain [45]. This debate concerns also the SIRT1 activators developed by Sirtris that are discussed in the following section.

Among the polyphenols that were studied for their effects on SIRT1, it is worth mentioning two catechins conjugated to a gallic acid portion, epicatechin gallate **8** and epigallocatechin gallate **9**, and the flavonol myricetin **10** (Figure 1). These natural polyphenolic compounds are readily oxidized in aqueous media, forming hydrogen peroxide. Therefore, they were tested in the presence of vitamin C, which acted as a stabilizer. Interestingly, these three natural compounds stimulated SIRT1 only under stabilizing conditions; conversely, without stabilization they inhibited SIRT1. This difference was attributable to the fact that polyphenols are readily oxidized in the aqueous assay medium, forming hydrogen peroxide, which probably is the cause of protein activity decrease, while when these compounds were tested in the presence of vitamin C, the H_2O_2 production was decreased. The importance of the stabilization of these compounds was confirmed by the observation that the corresponding derivatives of epicatechin gallate and epigallocatechin gallate without the gallic acid portion were not able to stimulate SIRT1, since the gallolyation of the polyphenolic scaffold prevented the conjugation to glucuronides and sulfates, thus increasing their bioavailability. In the same study, the authors stressed the importance of the metabolism for the activities of

polyphenols, taking as an example the case of quercetin, which was reported as a SIRT1 activator on the isolated protein. However, this compound did not affect SIRT1activity in human colon carcinoma HT29 cells, likely due to the formation of metabolite quercetin 3-*O*-glucuronide [46].

Structurally similar isoflavones daizein **11**, formononetin **12**, 7-hydroxy-*4H*-chromen-4-one (7-C, **13**) and 3-(2',4'-dichlorophenyl)-7-hydroxy-*4H*-chromen-4-one (DCHC, **14**) (Figure 1) were found to increase SIRT1 activity even at concentrations in the low micromolar range. These natural compounds were able to promote mitochondrial biogenesis and functionality by means of activation of PGC-1α, which is regulated by SIRT1 [47]. Moreover, daidzein and formononetin also promoted the expression of SIRT1.

Similarly, resveratrol was reported to promote mitochondrial biogenesis through a SIRT1-dependant deacetylation and consequent activation of PGC-1α. After resveratrol administration to mice, a significant increase in their aerobic capacity was observed, demonstrated by an increase in the oxidative type-muscle fibers, an enhanced resistance to muscle fatigue, and an increased tolerance to cold [48].

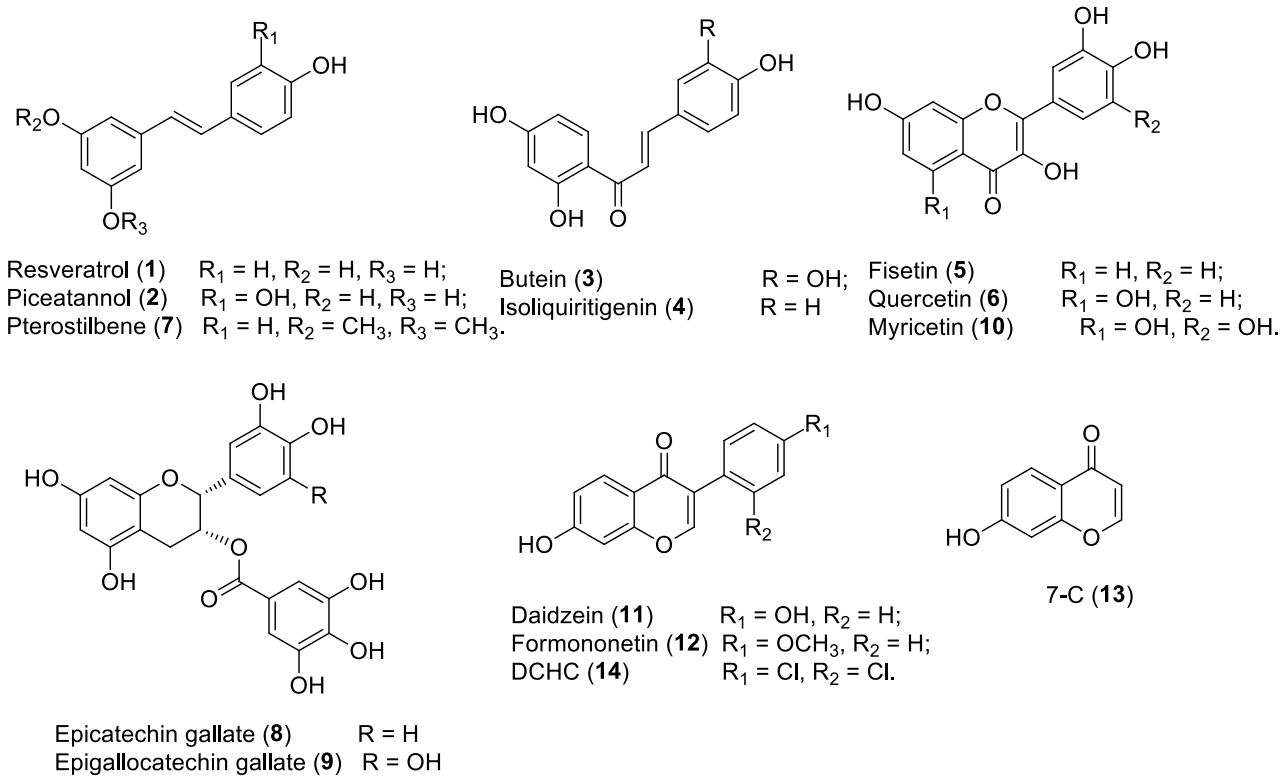


Figure 1. Structures of natural SIRT1 activators: resveratrol and flavonoids.

20(*S*)-Ginsenoside Rg_2 **15** (Figure 2) is a saponin, extracted from roots and rhizomes of *Panax ginseng*, that stimulated SIRT1 activity. In particular, two metabolites of 20(*S*)-Ginsenoside Rg_2, A and B (Figure 2), were identified after treatment of the ginsenoside with rat liver microsomes: they possess the same central structure of 20(*S*)-Ginsenoside Rg_2, but differ from it because they bear a substituted tetrahydrofuran ring instead of the aliphatic side chain on the cyclopentane ring, probably deriving from an initial epoxidation of the double bond followed by an intramolecular nuelophilic/ring-opening reaction. They proved to be more potent than the parent compound at the concentration of 20 μM. In fact, SIRT1 activity was increased up to 160-180% compared to control and, therefore, the metabolic conversion of 20(*S*)-Ginsenoside Rg_2 may be responsible of most of its biological activity [49].

A screening of compounds belonging to Traditional Chinese medicines identified Ginsenosides Rb_2 **16**, Rc **17**, F1 **18** (from *Panax ginseng*) and Schisandrin A **19** (from *Schisandra chinensis*) as activators of SIRT1 (Figure 2). Their effects against oxidative damage in H9c2 cardiomyocytes exposed to *tert*-butyl hydroperoxide was investigated and all the four natural compounds exerted a protective effect, by recovering the physiological oxygen consumption rate. Furthermore, pre-treatment of cardiomyocytes with these compounds reversed the decreased mitochondrial ATP production, prevented ROS formation, enhanced the activity of the mitochondrial antioxidant manganese superoxide dismutase (Mn-SOD) enzyme and increased the mitochondrial DNA content, thus suggesting a stimulation of mitochondrial biogenesis [50].

A further natural compound isolated from *Panax ginseng* leaves, the dammarane triterpene dammar-20(22),24-diene-3β,6α,12β-triol **20** (Figure 3), showed a promising activity as SIRT1 activator, increasing its activity up to 250% compared to control at the concentration of 20 μg/mL. The SIRT1 stimulating activity of this compound was confirmed in cell-based assays using human embryonic kidney HEK293 cell

line, in which it decreased p53 transcriptional activity and increased the intracellular NAD^+/NADH ratio [51].

Terpenylated coumarin **21** (Figure 3), isolated from *Ailanthus altissima*, was identified by a screening on natural products aimed at discovering SIRT1 activators. It activated SIRT1 in a dose-dependent manner in both enzymatic and cell-based (human embryonic kidney HEK293 cells) assays [52].

β-Lapachone **22** (Figure 3) is a natural *o*-naphthoquinone, originally extracted from the bark of the lapacho tree (*Tabebuia avellanedae*). β-Lapachone is reduced by NADH:quinone oxidoreductase (NQO1) thus generating NAD^+ from NADH. Therefore, β-Lapachone can modulate the NAD^+/NADH ratio, thus influencing SIRT1 activity, which requires NAD^+ as the cofactor. In fact, increased levels of NAD^+ promote SIRT1 enzymatic activity. β-Lapachone administration to mice displaying severe lipotoxic cardiomyopathy resulted in a reduced lipid accumulation, inhibition of heart failure and improvement of cardiac functionality [53].

The natural phenolic derivative paeonol **23** (Figure 3) is an active component of the Moutan Cortex, a traditional Chinese medicine used to alleviate the effects of cardiovascular diseases associated with oxidative stress, such as atherosclerosis and myocardial infarction. Paeonol is usually isolated from root bark of Chinese Peony tree. An enzymatic assay revealed the activating ability of paeonol on SIRT1 (about 2.5-fold increase compared to control, but less potent than resveratrol). This mechanism with the subsequent downregulation of the SIRT1 target protein p53 were considered responsible for the protective effect of paeonol against hydrogen peroxide-induced cell growth arrest of endothelial cells, since SIRT1 expression resulted to be decreased in these damaged cells. Pretreatment of human umbilical endothelial cells (HUVECs) with paeonol at 30 μM for 24 h reduced the number of senescent cells and increased cell proliferation [54].

Unfortunately, resveratrol and the other mentioned polyhydroxylated natural phytochemicals reported in Figure 1 showed a well-documented promiscuous action and, although studies of resveratrol activities and functions are still abundant in the literature, it is actually considered a pan-assay interference compound (PAINS) [55]. Similarly, the biological effects of polyphenols are attributed to their plethora of actions such as antioxidant, metal-ion chelating and free radical scavenging activities, together with their reported activities on many other signaling pathways. Therefore, these compounds will not be discussed further, since this review is focused on selective SIRT1activators.

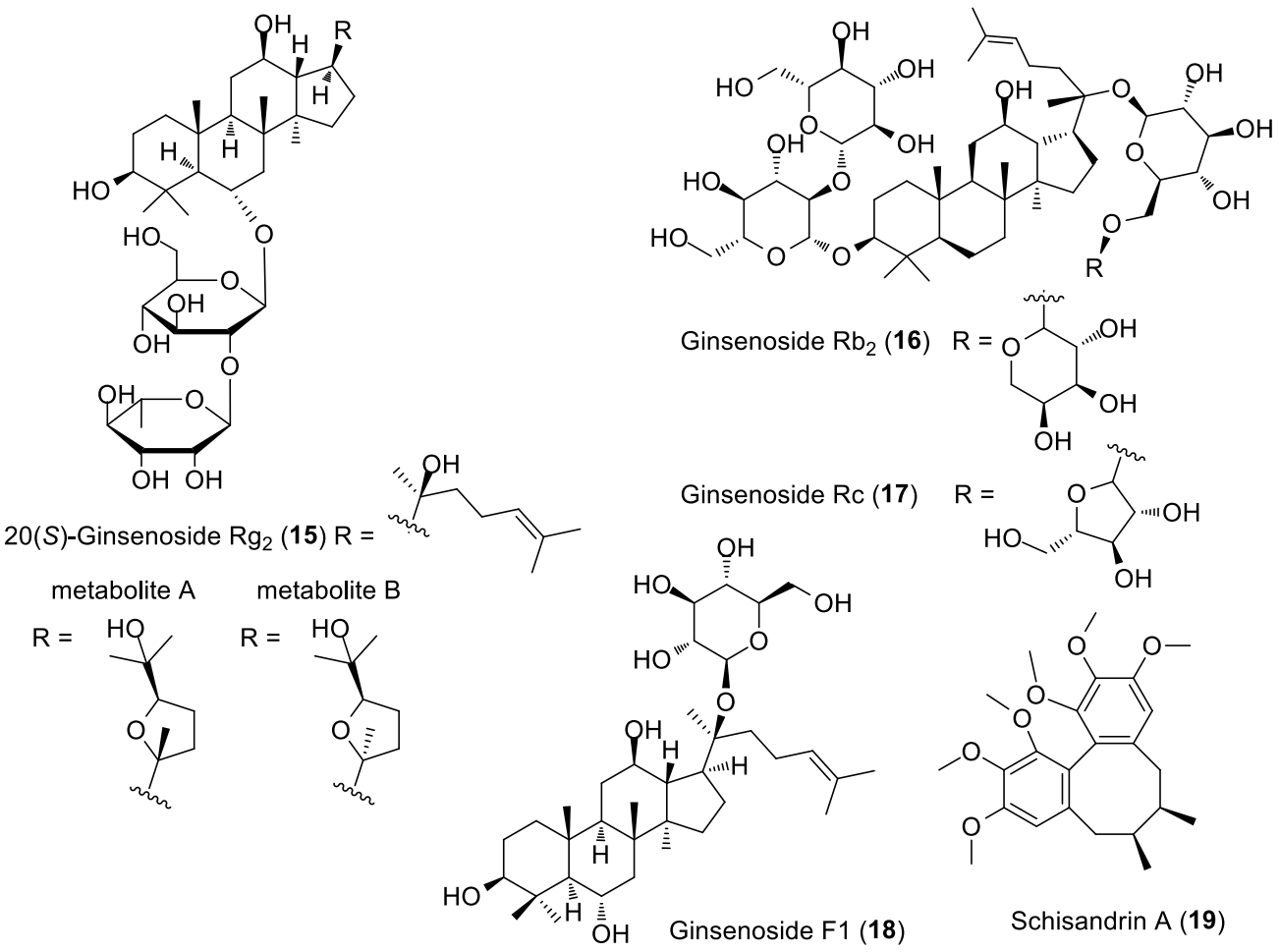


Figure 2. Structures of SIRT1 activators: Ginsenosides and Schisandrin A.

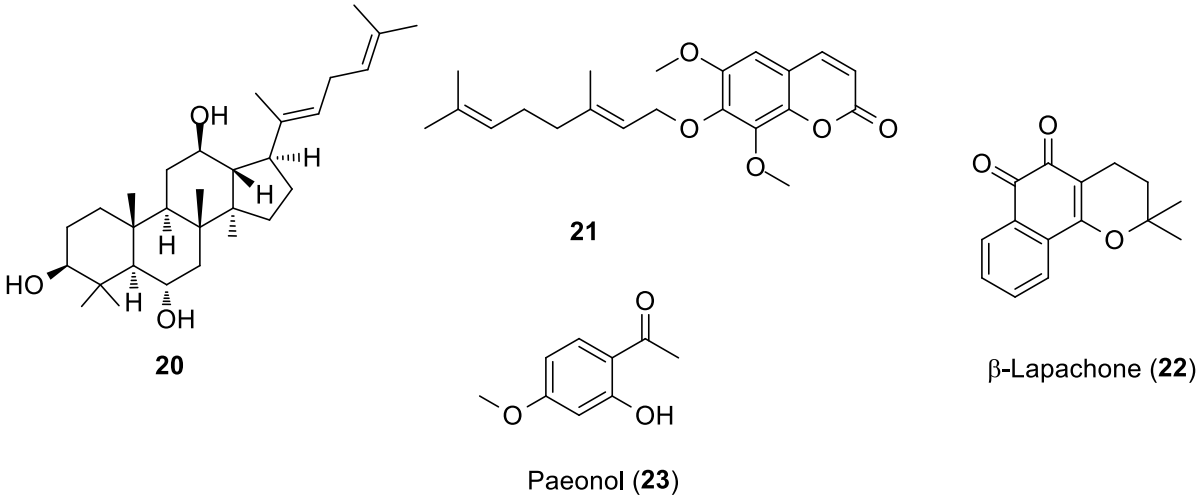
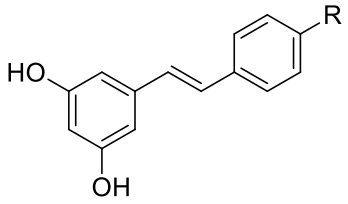


Figure 3. Structures of other natural SIRT1 activators.

2.2. *Synthetic activators*

A great interest for the discovery of new SIRT1 activators has so far led several research groups, both in academy and in pharmaceutical industry, to the synthesis of various chemical classes of compounds. Synthetic STACs are more potent than resveratrol and related polyphenols or than any other natural compounds tested thus far, and some of them are currently in human clinical trials.

The first effort to discover new synthetic SIRT1 activators started from the observation of the SIRT1-activating effects of resveratrol, together with the several limitations (low solubility, bioavailability and unspecific actions) associated with this natural compound. Therefore, Sinclair *et al.* developed a resveratrol-like series of stilbene analogues (**24**-**25**, Figure 4) in which the hydroxyl groups in the 3 and 5 positions as well as the *trans* configuration of the two aromatic rings of resveratrol were maintained, whereas the 4'-hydroxyl group was replaced by different groups. The presence of a thiomethyl substituent (compound **24**) stimulated SIRT1 activity by 18-fold (compared to resveratrol which increased SIRT1 activity 12-fold), leading to the more potent derivative of this class of compounds.



24 R = -SCH_3;
25 R = -$OCOCH_3$.

Figure 4. Structures of stilbene derivatives.

This thiomethyl derivative proved to be less cytotoxic than resveratrol in HEK293 cells. Compound **25** bears an ester group at the 4' position. Initially, this relatively large ester group seemed to be detrimental for the activity (SIRT1 increase of only 3-fold); however, compound **25** showed an improved stability in ethanol at a 2.5 mM concentration at room temperature when exposed to ambient light, with a half-life of about 10 days, compared to the parent compound resveratrol (half-life 4.5-5 days). This improved stability was reflected also in the ability of this compound to extend *Saccharomyces cerevisiae* lifespan: compound **25** enhanced the lifespan duration more efficiently than resveratrol, despite the lower potency *in vitro*, probably due to its longer effect duration [56].

The first example of SIRT1 activators structurally unrelated to resveratrol was realized by a screening and a subsequent structural optimization performed by Sirtris Pharmaceuticals (a GSK company), which led to the discovery of three imidazothiazole SIRT1-activators named SRT1460 (**26**), SRT1720 (**27**) and SRT2183 (**28**) (Table 1). Their potency was determined by using two parameters: $EC_{1.5}$, that is, the concentration of compound required to increase enzyme activity by 50%, and maximum activation, consisting in the percentage maximum activation achieved at the highest doses of the tested compound. All the compounds showed excellent $EC_{1.5}$ values in the low micromolar range (SRT1460 $EC_{1.5}$ = 2.9 μM) or even submicromolar range (SRT1720 and SRT2183 $EC_{1.5}$ = 0.16 and 0.36 μM, respectively) and maximum activation levels in the range of 296-781%. A series of different substituents were introduced in the amide portion of this central scaffold (cycloalkyl, variously substituted aromatic and heteroaromatic rings), as well as water-solubilizing amino groups were inserted in the C2 or C3 position of the imidazothiazole core (dimethylamino or piperazine moieties), but the presence of the 2-quinoxaline group as the amidic substituent with the piperazine at the C3 position of SRT1720 currently represents the best combination to achieve an excellent SIRT1 activating property. Moreover, all the compounds were selective for SIRT1 *versus* similar isoforms SIRT2 and SIRT3 ($EC_{1.5}$ > 300 μM), with the exception of SRT1720, which showed an $EC_{1.5}$ non-negligible value of 37 μM for

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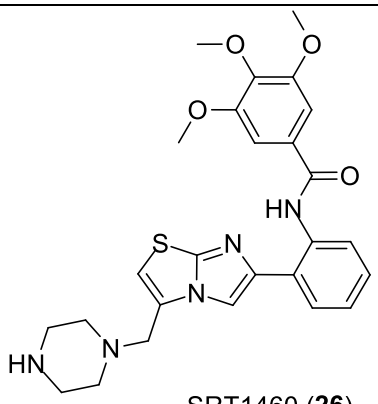
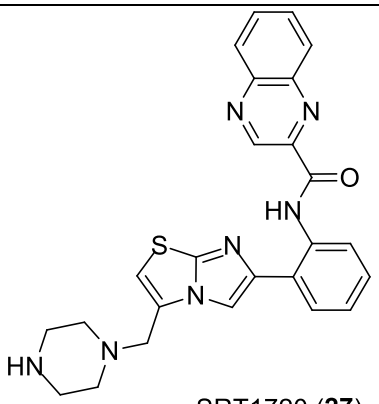
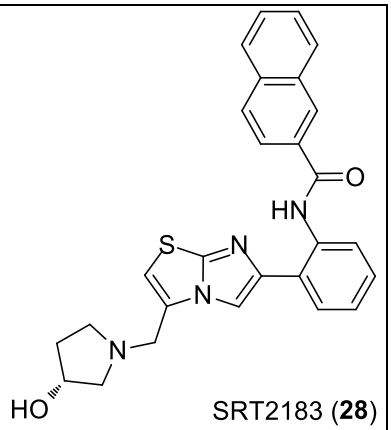
SIRT2. The activity of these compounds was confirmed in functional *in vitro* assays in the human osteosarcoma U2OS cells, in which the decrease of the acetylation state of p53 was measured, and the fact that the deacetylation effect was mediated by SIRT1 was further confirmed by using a SIRT1 inhibitor, which actually blocked their effect. Surprisingly, isothermal titration calorimetry studies did not reveal any detectable binding of SRT1460 to purified SIRT1, whereas in the presence of the peptidic substrate it was observed a binding till the saturation. This unexpected behavior was explained by supposing that the binding of an acetylated substrate induces a conformational change of the protein that leads to the exposure of an allosteric binding site where the activator can bind, thus enhancing the catalytic activity. This hypothesis was confirmed by identifying an allosteric binding site located in the *N*-terminal portion of the catalytic domain (amino acid sequence: 183-225). Three *in vivo* models of type 2 diabetes were used to assess the efficacy of these compounds *in vivo*: diet-induced obesity (DIO) mice, genetically obese (*Lep*$^{ob/ob}$) mice and Zucker *fa/fa* model. The compound with the best pharmacokinetic profile for *in vivo* evaluation (SRT1720) was tested by oral administration to assess its therapeutic utility, since SRT1720 showed good oral bioavailability both in mice and rats. In the first two models (diet-induced and genetically obese animals), SRT1720 (100 mg/Kg once daily) normalized the plasma glucose level, improved the insulin sensitivity and increased the mitochondrial capacity, without any signs of toxicity after 10 weeks of dosing. In the last animal model (Zucker *fa/fa* model) consisting of genetically obese mice to study insulin resistance, SRT1720 succeeded in improving insulin sensitivity in skeletal, adipose and liver tissues, and it decreased the hepatic gluconeogenesis. In order to assess the presence of possible off-targets, compound SRT1720 was evaluated on a panel of 40 receptors and the main isoforms of cytochrome P450 enzymes, but it showed no significant activities at the highest tested concentrations [57, 58].

Table 1. SIRT1 activity and structure of SRT1460, SRT1720 and SRT2183.

	 SRT1460 (**26**)	 SRT1720 (**27**)	 SRT2183 (**28**)
$EC_{1.5}$ (μM)[a]	2.9	0.16	0.36
EC_{50} (μM)[b]	21.9 ± 1.1	8.8 ± 1.3	-
max act. (%)[c]	447	781	296

[a] $EC_{1.5}$ is the concentration of compound required to increase enzyme activity by 50% [57];

[b] EC_{50} values determined from activation dose-response curves using the following equation: $v_x/v_0 = b + (RV_{max} - b)/(1 + EC_{50}/[X]_0$, where v_x/v_0 is the ratio of the reaction rate in the presence (v_x) versus absence (v_0) of activator (X), RV_{max} is the relative velocity at infinite activator concentration, EC_{50} is the concentration of activator required to produce one-half RV_{max} and b is the minimum value of v_x/v_0. Resveratrol was used as control: EC_{50} = 29.5 ± 1.1 μM [59];

[c] max act. is the percentage maximum activation achieved at the highest doses of tested compound. Resveratrol was used as control: $EC_{1.5}$ = 46.2 μM, max act. = 201% [57].

SRT2183 was also tested in renal medullary interstitial cells of the kidneys, which are usually exposed to high oxidative stress and overexpress SIRT1. SIRT1 activation by SRT2183 improved cell survival in response to oxidative stress. The mechanism by which SIRT1 activation protects these kidney cells from injury was investigated and it was observed that SRT2183 promotes cyclooxygenase-2 (COX2) expression, thereby COX2-derived PGE_2 exerts an antioxidant effect on these cells. This way. SRT2183 can minimize or prevent renal damage induced by oxidative stress [60].

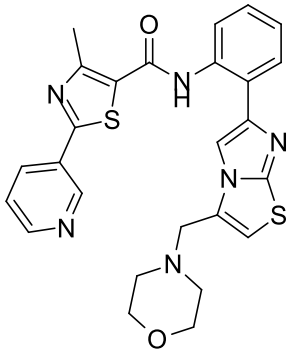
SRT1720 is one of the most studied SIRT1 activators and one of its characteristic therapeutic effects is to mimic low energy levels by activating metabolically important targets such as AMPK, FOXO1 and PGC-1α, through SIRT1-mediated deacetylation. Daily oral administration of SRT1720 to mice fed with a high-fat diet prevented fat accumulation, thus hindering weight gain and obesity,

without altering the intestinal absorption of nutrients, but rather by acting on glucose homeostasis, insulin sensitivity and fatty acid oxidation. However, these *in vivo* effects were observed at high doses (500 mg/kg/day) despite the high affinity of this compound for SIRT1, probably because of its rapid metabolism which determined a low bioavailability [61]. Different studies were in agreement to confirm that SRT1720 mimics calorie restriction, which results in an improvement of glucose and insulin homeostasis in several type 2 diabetes mouse models, and in a reduction of the inflammatory signaling pathways [62]. Similarly, SRT1720 was able to extend both mean and maximum lifespan of adult obese mice, accompanied by many health benefits, such as, an improvement of the hepatic and pancreatic dysfunctions induced by high-fat diet, an increased insulin sensitivity and glucose homeostasis, and enhanced motor function and metabolism [63]. SRT1720 determined positive effects also in mice fed a standard diet at the dose of 100 mg/kg body weight, such as, extension in lifespan, delayed onset of age-related metabolic diseases, and improved general health [64]. At the cardiovascular level, SRT1720 improved endothelial function in old mice, by enhancing COX-2-mediated vasodilation, thus repairing endothelial dysfunction. A direct correlation with SIRT1 activity of this compound was demonstrated by observing that SRT1720 normalized protein expression and activity of SIRT1 in arteries of aged mice [65]. SRT1720, when administered to C57BL/6 mice *via* tail vein injection, induced a decreased acetylation of PGC-1α in heart homogenates, thus confirming its involvement at the myocardial level. Moreover, SRT1720 proved to trigger the transport of SIRT1 from the cytoplasm into the nucleus, by promoting its sumoylation. Importantly, SRT1720 reduced myocardial infarction in aged hearts, by means of a reduction of apoptosis induced by ischemia/reperfusion insult [66]. SRT1720 was used as a representative SIRT1 activator to understand the link between SIRT1 and atherosclerosis: SIRT1 is involved in the prevention of atherosclerosis, since a low expression of SIRT1 was found in high glucose and high lipid environments, representative of the blood of patients with atherosclerosis. Liver X receptor (LXR), which usually behaves as a cholesterol sensor to protect the organism from cholesterol accumulation, is deacetylated and, consequently, activated by SIRT1. SRT1720 leads to SIRT1 activation and subsequent enhances expression of LXR and of its target genes, thus stimulating cholesterol efflux from cells. Moreover, SIRT1 was found to inhibit the expression and the activity of the transcription nuclear factor κB (NF-κB), thus reducing the production of pro-inflammatory factors [67]. SRT1720 was tested in monosodium glutamate mouse model (MSG mice), which is characterized by obesity and insulin resistance. A dose of 200 mg/kg body weight of SRT1720 in MSG mice resulted in a reduction of fat accumulation in the liver, as well as in an improvement of liver dysfunction, without influencing body weight or food intake. Expressions of genes involved in hepatic lipid synthesis were reduced. The reduction in lipid synthesis and accumulation caused by SRT1720 treatment may be useful for the treatment of the nonalcoholic fatty liver disease (NAFLD), which consists in an abnormal liver metabolism often observed with insulin resistance and metabolic syndrome [68]. In renal ischemia-reperfusion induced kidney injury, SRT1720-promoted SIRT1 activation exerted a protective effect and decreased renal injury by stimulating mitochondrial biogenesis and decreasing ROS formation and inflammation [69, 70].

The debate about the binding mechanism of resveratrol concerned also SRT1460, SRT1720 and SRT2183: all the three synthetic compounds were unable to bind substrates lacking a fluorophore and proved to directly interact with fluorophore-containing peptide substrates, even in the absence of SIRT1. Sirtris compounds were tested on two native full-length substrates, p53 (the most widely studied SIRT1 substrate) and acetyl-CoA synthetase1 (AceCS1, an optimal substrate due to its single site of acetylation) and they had no effect on SIRT1 deacetylating activity. Moreover, Pacholec *et al.* further discredited these compounds, by affirming that they were not able to improve the metabolism and the glucose levels of mice fed with a high-fat diet and that their activity was promiscuous, since they interacted with many different receptors and enzymes [71]. A completely contrasting evidence for these three compounds was reported in the same year by Dai *et al.*, who rejected the hypothesis that SIRT1 can be activated only if peptide substrates bear a fluorophore, stating that, on the contrary, SIRT1 deacetylation can be accelerated also in the presence of unlabeled natural substrates. The authors of this study did not observe any particular affinity of the activators towards the TAMRA-peptide substrate (TAMRA is tetramethyl-6-carboxyrhodamine) and the lack of the TAMRA portion did not seem to be mandatory for SIRT1 activation by Sirtris compounds [72].

SRT2104 (**29**, Figure 5) is a SIRT1 activator initially developed by Sirtris Pharmaceuticals (a GSK company), possessing the same imidazothiazole central scaffold as SRT1460, SRT1720 and SRT2183, and currently under development at GlaxoSmithKline Pharmaceuticals. SRT2104 was studied in a phase I clinical trial in elderly volunteers administered up to 2.0 g/day for 28 days. SRT2104 demonstrated to be well tolerated and it decreased cholesterol and triglyceride levels and increased the mitochondrial oxidative phosphorylation, which are all parameters that depend on SIRT1 activation [73]. This compound was evaluated in four phase 1 clinical studies in healthy adults to assess its safety, tolerability and pharmacokinetics and it was well tolerated without any adverse reaction. It was characterized by a poor absorbtion following oral dosing as a suspension due to its scarce solubility. The optimal dosing strategy to maximize the exposure was observed at the 2.0 g dose for 7 days [74]. A study on the effect of SRT2104 supplementation on health and lifespan in mice fed with standard diet was performed to determine the long-lasting effects elicited by this compound. SRT2104 improved survival of treated mice and increased mean and maximum lifespan, without affecting caloric intake or voluntary activity. Moreover, it delayed the onset of age-related diseases. Similar to other SIRT1 activators, SRT2104 increased mitochondrial content and suppressed the inflammation pathways [75]. A therapeutic application of SRT2104 to treat Huntington's disease was considered,

because this compound exerted a protective effect in this neurodegenerative disease. It was observed that SRT2104 penetrates the blood-brain barrier and, once in the brain, it reduces brain atrophy and leads to improvements in motor function in a mouse model of Huntington's disease; moreover, the survival of treated animals resulted to be increased following SRT2104 administration [76]. SRT2104 was orally administered to healthy cigarette smokers in a clinical trial (2.0 g/day for 28 days). This compound proved to be safe and well tolerated and it induced a reduction of blood cholesterol level, without affecting any other cardiovascular or endothelial functions. This improvement in lipid profile reduced the risk factor for atherosclerosis and coronary heart disease, but the exact mechanism behind this positive effect still needs to be determined [77]. Oral administration of SRT2104 in healthy cigarette smokers and people with type 2 diabetes elicited a significant reduction in arterial stiffness, which is considered as a measure of cardiovascular risk [78].



SRT2104 (**29**)

Figure 5. Structure of SRT2104.

SRT3025 developed at Sirtris Pharmaceuticals (a GSK company) (undisclosed structure, at present in a phase I clinical trial for type-2-diabetes-mellitus) was tested in in a mouse model of atherosclerosis and in hepatocyte culture, in which it decreased plasma levels of LDL-cholesterol and total cholesterol and reduced atherosclerosis. The biochemical mechanism of these effects was explained considering that SRT3025-mediated SIRT1 activation attenuated proprotein convertase subtilisin/kexin type 9 (Pcsk9) secretion from hepatocytes *in vitro* and lowered plasma levels of Pcsk9 *in vivo*. As a consequence, hepatocyte hepatic LDL receptor (Ldlr) expression and activity were increased, thus promoting a decrease in plasma LDL-cholesterol and atherosclerotic plaques [79]. SRT3025 in an *in vivo* model of Fanconi anemia (a genetic bone marrow failure syndrome) increased hematopoietic stem and progenitor cells, platelets and white blood cells. Surprisingly, these effects were not abrogated by SIRT1 deletion, suggesting that the beneficial effects were not mediated by SIRT1, but rather this SIRT1 activating compound acted indirectly via extra-hematopoietic effects [80]. Moreover, SRT3025 together with SRT1720 and SRT1460, exerted antiproliferative effects in human pancreatic cancer, both in cell cultures and in xenograft experiments, and the mechanism underlying cell death involved lysosomal function. This study defined a positive effect exerted by this series of compounds in cancer, despite the controversial role of sirtuins in cancer. Promising combination effects with standard drugs used for pancreatic cancer, such as gemcitabine and paclitaxel, were observed. In fact, pancreatic cancer cell survival was more efficiently arrested in the presence of combinations of these drugs [81].

Other structural classes of SIRT1 activators were discovered by Sirtris Pharmaceuticals and the best compounds **30**-**32** are reported in Figure 6. All the compounds are based on a central bicyclic heteroaromatic scaffold (oxazolopyirdine, benzimidazole or azabenzimidazole cycle) with a phenyl ring linked to the five-membered ring. The substituents present on the peripheral phenyl ring that gave the best results in terms of activation potency are methoxy-substituted benzamides, quinoxaline amides and piperazine moieties, thus strictly resembling the groups that are present in the imidazothiazole series. However, for these compounds no potential therapeutic applications were further investigated. Oxazolopyridine **30** and azabenzimidazole **32** displayed the same $EC_{1.5}$ value of 0.5 μM, with a similar activation percentage in the range 220-270%. Differently, the more active benzimidazole **31** showed an $EC_{1.5}$ value of 0.4 μM, reaching an excellent 820% of maximum activation [82].

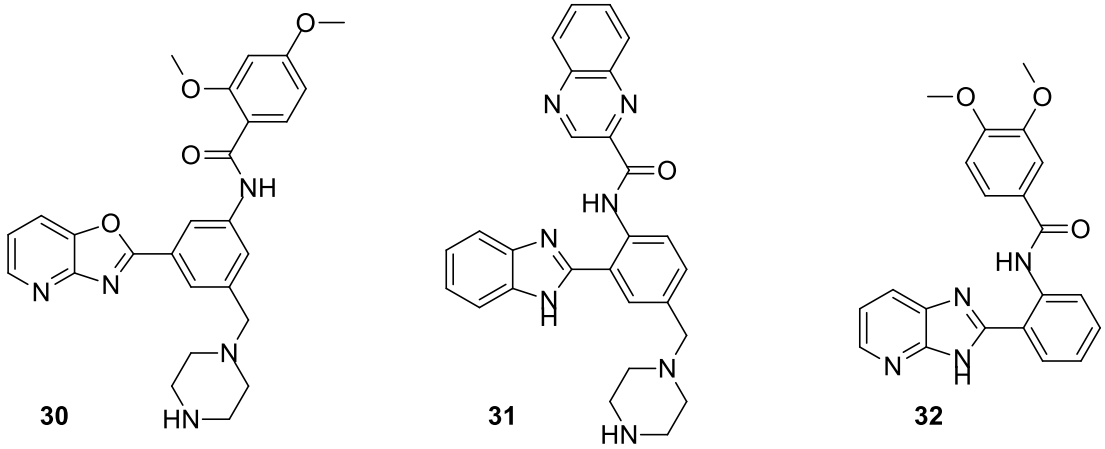
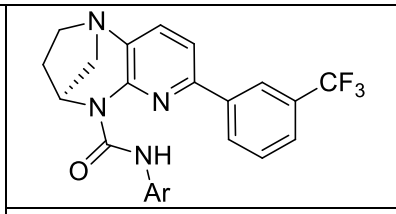
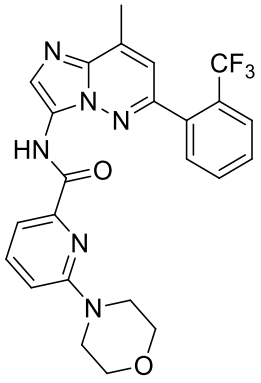
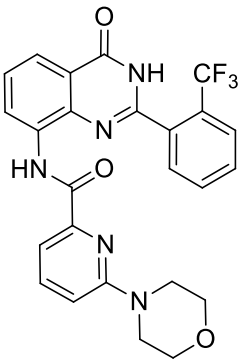


Figure 6. Structures of three representative examples of SIRT1 activators belonging to oxazolopyridine, azabenzimidazole and benzimidazole classes.

Dai *et al.* of Sirtris research group developed an engineered *h*SIRT1 (mini-*h*SIRT1) that was able to perform lysine deacetylation and to be catalytically activated by compounds in the same manner as the full-length enzyme. This protein represents the first example of a biochemically functional SIRT1 that can be characterized in order to understand the binding of STACs to the protein. A series of ureic and amidic SIRT1 activators were developed and the most active derivatives are reported in Table 2 (compounds **33-37**). All of them displayed EC_{50} values in the nanomolar range and maximum activation values greater than 10, with the exception of compound **34**. Compound **33** was co-crystallized with this protein and it was found to be located in the *N*-terminal domain of mini-*h*SIRT1. Therefore the STAC-binding domain was identified in the *N*-terminal domain of this enzyme, whereas the central part is responsible of the catalytic activity and the *C*-terminal segment stabilizes the catalytic domain. Compound **33** establishes mainly lipophilic interactions with the protein, forming only one hydrogen bond between the carbonyl oxygen and the carboxamide nitrogen of Asn226 in the STAC-binding site, which can be described as a superficial cavity containing an off-center hydrophobic pocket in which the trifluoromethyl group of the peripheral phenyl ring of compound **33** was located. The hydrophobic depression observed in the crystal structure justified the flatness and the high hydrophobicity typical of most activators. Site-directed mutagenesis studies were performed with the aim of identifying the essential residues of the STAC-binding pocket for the activity, comparing the activation potencies of the compounds reported in Table 2 on the mutant versus wild-type mini-*h*SIRT1. These studies helped to identify a role for Glu230, whose negative charge was important for stabilizing the activated conformation, and the positively charged Arg446 could represent an electrostatic partner of Glu230, thus stabilizing the activated conformation [83].

Table 2. SIRT1 activity and structure of ureic and amidic activators **33-37**.

	 Ar **33**	Ar **34**	Ar **35**	 **36**	 **37**
EC_{50} (μM)[a]	0.30	0.48	0.77	0.77	0.44
RV_{max}[b]	10.5	6.28	14.9	14.7	12.2

[a] EC_{50} values determined from activation dose-response curves using the following equation: $v_x/v_0 = b + (RV_{max} - b)/(1 + EC_{50}/[X]_0$, where v_x/v_0 is the ratio of the reaction rate in the presence (v_x) versus absence (v_0) of activator (X), RV_{max} is the relative velocity at infinite activator concentration, EC_{50} is the concentration of activator required to produce one-half RV_{max} and b is the minimum value of v_x/v_0;

[b] Maximum activation values (RV_{max}) determined from activation dose-response curves, using the same equation used to calculate EC_{50} [83].

The activation mechanism of STACs was further investigated by using the already described SRT1460 and the pyridinethiazole derivative **38** (EC_{50} = 1.0 ± 1.1 μM) from Sirtris library (Figure 7). Their effects were tested on two SIRT1 substrates, PGC-1α-K778 and FOXO3a-K290, which contain aromatic and hydrophobic residues near the acetylated lysine. It was observed that these hydrophobic moieties facilitate SIRT1 activation by these compounds, hence these compounds behave according to an "assisted allosteric activation" mechanism. Probably, naturally occurring hydrophobic amino acids could be efficiently mimicked by bulky and hydrophobic fluorophores that are present in many commercially available substrates used for enzymatic assays, thus solving the long-lasting debate about the ability of STACs to activate only fluorophore-tagged substrates. Furthermore, the critical role of Glu230 for activation was confirmed [59].

A series of pyrroloquinoxaline (Figure 7) derivatives were developed at S*BIO Pte Ltd company by an HTS of commercial compound libraries, previously filtered on the basis of drug-likeness properties. A fluorescence-based enzymatic assay showed relevant activation potencies on

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recombinant SIRT1 of three compounds, and the structure of the most active activator **39** is reported in Figure 7. When tested at a 10 μM concentration, it increased the signal of 216.5 ± 61 %, compared to control. Since SIRT1 deacetylates the nuclear peroxisome proliferator-activated receptor gamma (PPARγ) in adipocytes, promoting lipolysis, the pyrroloquinoxaline was tested in cell-based adipogenesis differentiation assays. In these assays, this compound decreased the size and the number of cellular fat deposits, therefore its ability to affect fat mobilization in adipocytes should be mediated by SIRT1 activation. Moreover, an antiflammatory effect was observed by measuring the amount of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) released by human leukemia THP-1 cells: TNF-α was reduced by about 2-fold at 20 μM and 5-fold at 60 μM [84].

A new synthetic strategy led to the synthesis of a series of dihydropyrroloquinolinones (Figure 7) and, among them, compound **40** showed a good activation level of SIRT1 at the concentration of 10 μM (about 2-fold compared to control) and modeling studies in a homology model of *h*SIRT1 were performed to explain the binding disposition of this compound [85].

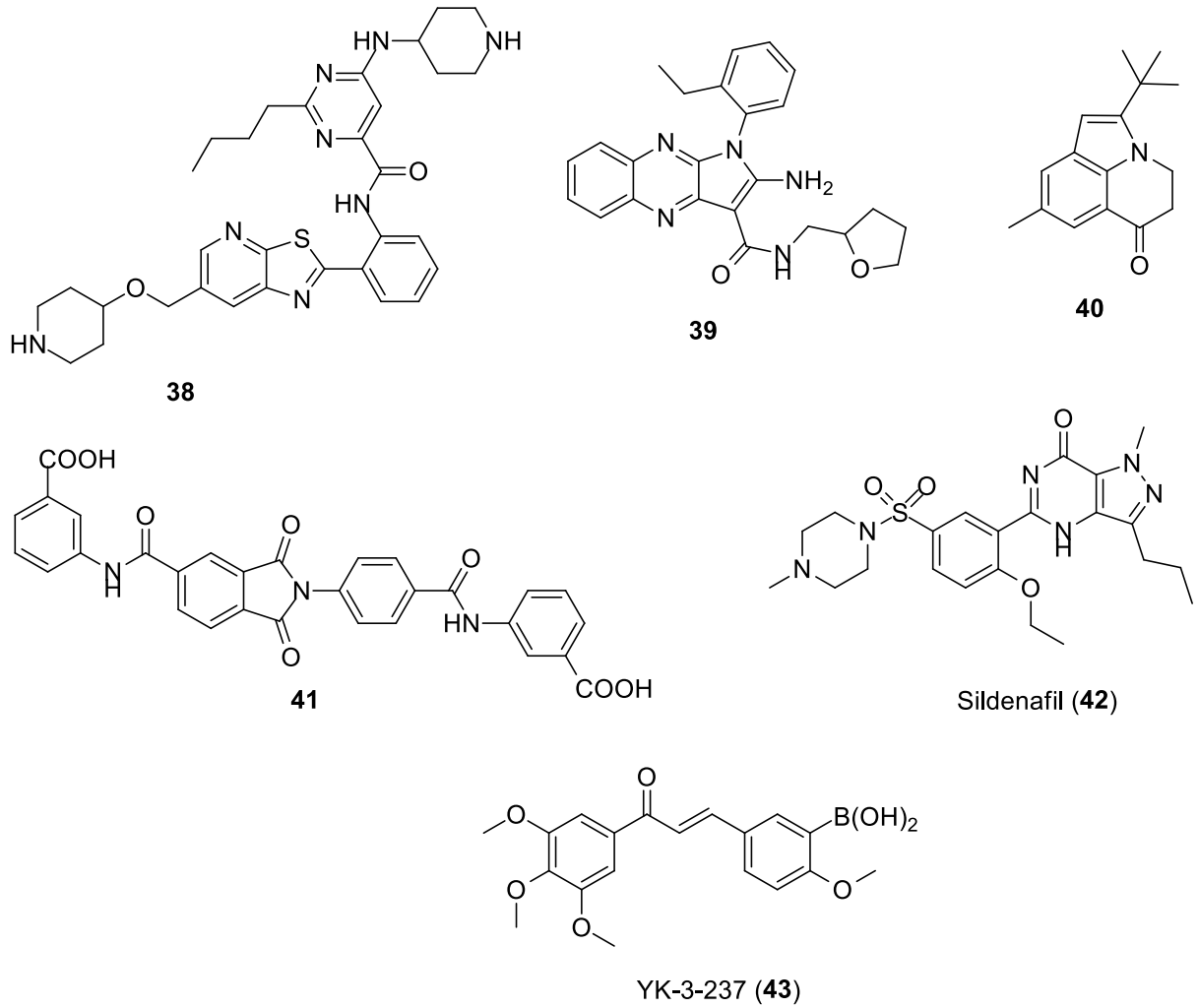

Figure 7. Structures of synthetic SIRT1 activators.

A virtual screening study of the Asinex database by using a homology model of the allosteric binding site of human SIRT1 identified phthalimidic derivative **41** (Figure 7) as a SIRT1 activator. This molecule interacts with several aromatic and charged residues in the allosteric binding site of the enzyme by electrostatic and stacking interactions (Ser174, Asp175, Arg167, Arg181 and Gln190), and completely occupied the cavity of its binding pocket. This compound was later tested in enzymatic assays and it caused an increase in the fluorescence especially at the highest tested concentrations (25 and 50 μM), which was even greater than that induced by resveratrol. Its therapeutic utility as an anti-obesity agent was assessed by testing this activator in adipocytes, in which it reduced the lipid content by more than 50%. The pharmacokinetic parameters were predicted and the compound displayed a good partition coefficient, a very good aqueous solubility, but unfortunately its cell permeability through Caco-2 cells was scarce as well as its oral absorption, probably due to its bulky structure and high molecular weight (greater than 500 Da). In conclusion,

despite the promising activity on the isolated enzyme and in cell-based assays, the unfavorable physico-chemical properties of **41** precluded its further development [86].

The well-known phosphodiesterase-5 inhibitor Sildenafil (**42**, Figure 7) exerted a protective effect against myocardial ischemia/reperfusion injury and it was hypothesized that this effect could be due to SIRT1 activation. *In vivo* studies evaluated the effects of sildenafil in ischemia/reperfusion injured mice, in which the myocardial infarct size was reduced and the effect was abolished by a SIRT1 inhibitor. The specific effect of sildenafil on SIRT1 activity was tested in homogenized heart samples: sildenafil at the low dose of 0.7 mg/Kg increased SIRT1 activity 24 h after treatment, whereas on isolated cardiomyocytes 1 μM sildenafil rapidly increased SIRT1 after only 1 h incubation, proving to be more effective than resveratrol. In these assays, SIRT1 protein expression resulted intensified, consequently the influence of sildenafil on protein expression contributed to its SIRT1-mediated cardioprotective effect [87].

Synthetic derivative YK-3-237 (**43**, Figure 7) was originally synthesized as a boronic acid chalcone derivative able to induce cell death in a wide panel of cancer cells [88]. Later, the ability of YK-3-237 to exert an antiproliferative effect was investigated especially in the breast cancer cell lines expressing mutant p53, which is a pro-tumorigenic factor. Western blot analysis revealed that 1μM concentration of this compound reduced mutant p53 expression in all the breast cancer cells, as well as the acetylation level of K382 of both mutant and wild-type p53. Considering that p53 is a well-known target of SIRT1, an enzymatic assay confirmed that YK-2-237 interfered with mutant p53 by activating SIRT1 more efficiently than resveratrol. YK-2-237 is not selective for SIRT1, since it also activates SIRT2 with a similar potency [89].

A screening by enzymatic assay using a 4-amino-7-methylcoumarin-labeled peptide substrate identified a series of diaryl acylhydrazones that are able to activate SIRT1. Then subsequent structural optimization steps led to the identification of the best activators belonging to this chemical class (compounds **44-45**, Figure 8). The bromo-substituted naphthohydrazone **44** displayed an $EC_{1.5}$ of 2.7 μM and a maximum activation of 647%, and the replacement of the bromine atom on the naphthalene ring by a methoxy group, as in compound **45**, maintained approximately the same maximum activation (624%), but the $EC_{1.5}$ value was greatly improved, reaching the submicromolar range (0.9 μM). Both the activators proved to be selective for SIRT1 against the similar isoforms SIRT2 and SIRT3. Then, compound **44** was tested by using an unlabeled acetyl peptide and the presence of the deacetylated peptide was measured by HPLC analysis, but in these assay conditions compound **44** did not enhance SIRT1 deacetylase activity. In order to explain these controversial data, a homology model of SIRT1 with the coumarin-labeled peptide was built and used to predict the binding mode of compound **44**. The napthtalene aromatic scaffold of compound **44** establishes a π-π stacking interaction with the fluorophore. On the other side of the molecule the *p*-hydroxy group of the dibromo-dihydroxyphenyl ring interacts with the side chain of Gln345 and the *o*-hydroxy group interacts with the substrate by H-bonds. Also the central imino group seems to be involved in the interaction with the substrate. The presence of the bulky and rigid fluorophore expands the substrate binding domain, by disrupting the interactions between the substrate and SIRT1. In this context, compound **44** behaves as a sort of mediator, since it compensates for the enlarged cavity created by the fluorophore, by promoting a tighten binding of the substrate with the protein: these actions result in an improvement of the affinity of the substrate to the enzyme, thanks to a stabilization of the complex which facilitates the catalytic mechanism. Compound **44** was tested at 100 μM in breast MCF-7 cancer cells, in which it proved to be sufficiently stable. The functional activity was confirmed in cell-based assays, although the compound lost its activation ability at low concentrations, probably due to its limited stability and cellular uptake [90].

The class of 1,4-dihydropyridine-based SIRT1 activators (Figure 8) was developed by the research group of Prof. A. Mai. The presence of substituents at the *N* position and at the 3,5 positions of the dihydropyridine ring, bearing a phenyl ring at the C4 position, determined the type of effect on SIRT. A benzyl group at the *N* position conferred activating properties to the resulting compounds **46**, **47** and **48**, regardless the presence of ethyl ester, amide or carboxylic acid groups at the 3,5 positions. The resulting compounds were not selective for SIRT1, since they were tested on SIRT1, SIRT2 and SIRT3 displaying similar activation potencies. In the SIRT1 assay, compounds **46** and **47** gave EC_{150} values of about 1 μM (EC_{150} indicates the effective concentration necessary to increase the enzymatic activity of 150%), whereas **48** was less potent (EC_{150} = 36 μM). A functional assay in human leukemia U937 cells confirmed the previous assays, showing a marked deacetylation of the SIRT2 target α-tubulin. In the same cell line, these compounds induced a cell cycle arrest, but surprisingly they did not exert the same effect in the breast cancer MCF-7 cells. They were tested in primary human mesenchymal stem cells (*h*MSC), where they proved to reduce the percentage of senescent cells of about 30-40%. Finally, only compound **48** increased mitochondrial function in murine myoblasts [91].

The promising results obtained with the first series of 1,4-dihydropyridine derivatives led to a further development of this chemical class. The phenyl ring at the C4 position was later replaced by five-membered aromatic rings, such as a 2-furyl or a 3-thienyl heterocycle in compounds **49** and **50** (Figure 8), respectively, or maintained as in compound **51** (Figure 8). The *N*-benzyl group of the previous series was maintained (compounds **49** and **50**) or replaced by a benzoyl moiety (compound **51**). These three new compounds proved to be more potent than the parent diethyl ester analog **46**, which increases SIRT1 activity of 217%: the presence of the benzoyl groups instead of the benzyl group led to a 244% activation (compound **51**), whereas maintaining the *N*-benzyl part and varying the heteroaromatic ring at the C4 led to a

marked increase of activation potency of 294% in the case of the introduction of a furan ring as in compound **49**, or reaching a 404% with a thiophene portion as in compound **50**. Nitric oxide synthase eNOS is a SIRT1 target that, after deacetylation, leads to NO release in endothelial cells. Therefore, the three reported compounds were tested in human keratinocyte HaCaT cells to determine if they could influence NO release. All of them induced an evident NO release and this effect was confirmed to be SIRT1-mediated by measuring the high SIRT1 activation induced by these compounds in the cellular context. These effects were abolished by the co-administration of a SIRT1 inhibitor and a AMPK inhibitor, thus demonstrating the involvement of the SIRT1/AMPK signaling pathway. In a mouse model of skin repair, they showed the capability to promote wound healing, when topically administered. Moreover, compounds **50** and **51** simulated mitochondrial biogenesis in mouse myoblasts. In order to exclude any possible off-target antagonistic effect on calcium channels, due to the 1,4-dihdryopyridine scaffold, compounds **50** and **51** were tested in guinea-pig isolated vascular and nonvascular smooth muscles, in order to assess the possible muscle relaxant effect: they proved to be completely inactive in this assay and, therefore, devoid of any calcium antagonist character. Compound **52** was synthesized as a water soluble analogue of compound **46**, bearing a (4-methylpiperazine)methyl group in *para* position of the C4-phenyl ring in its hydrochloride salt form and, despite its slightly lower activity as SIRT1 activator in enzymatic assays (152%), it exerted a good cytotoxic activity in a panel of cancer cell lines with IC_{50} values ranging from 8 to 35 μM, whereas compound **51** was less potent (IC_{50} values lower than 100 μM), displaying a comparable IC_{50} value (22 μM) only in colon cancer LOVO cells, probably due to its lower water solubility [92].

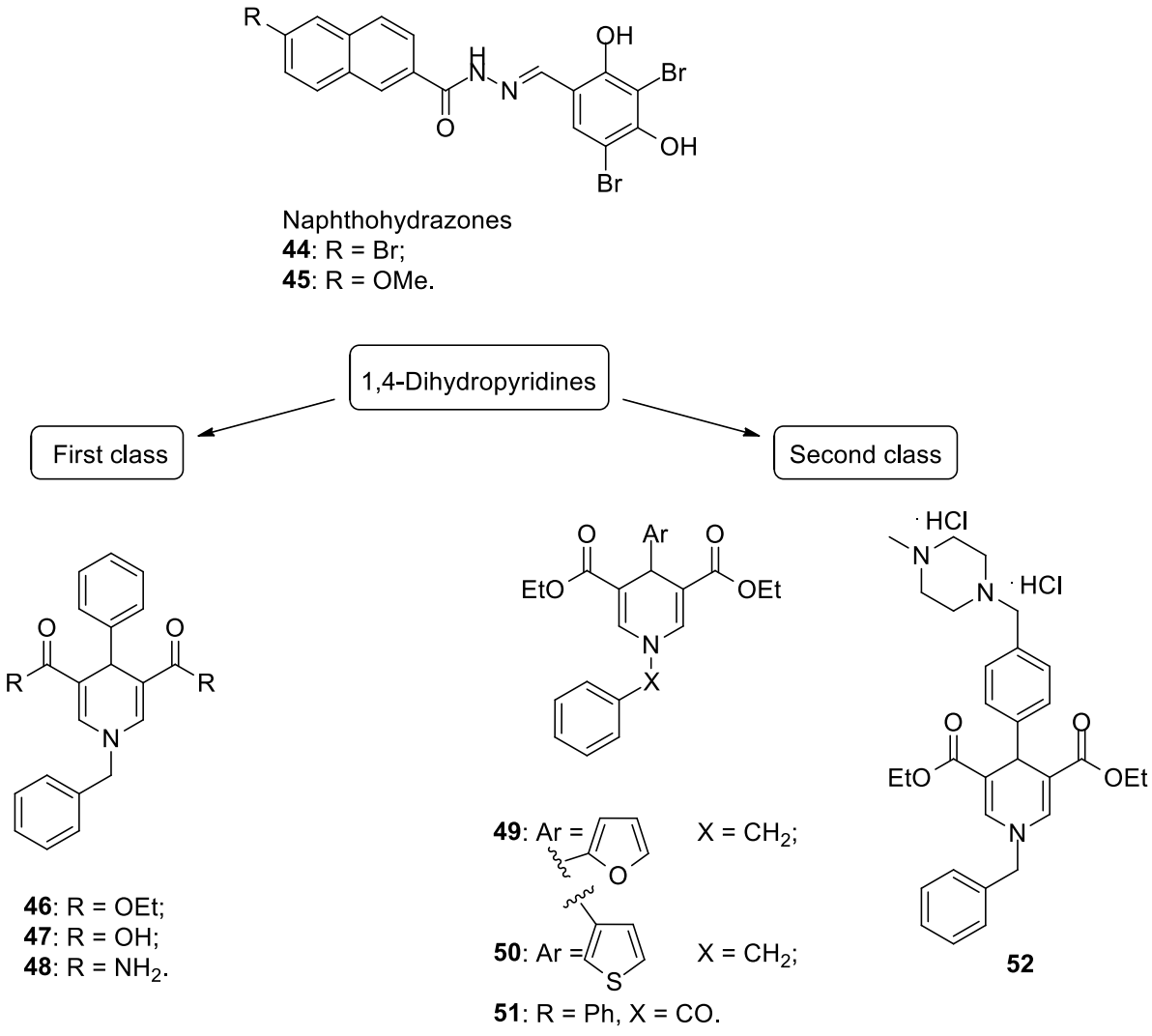

Figure 8. Structures of synthetic SIRT1 activators: naphthohydrazones and dihydropyiridines.

2.3. *Compounds increasing SIRT1 expression*

There is a further group of compounds that are able to influence SIRT1 activity by only increasing the expression of this protein. Most of them are natural compounds, but some examples of synthetic derivatives are also reported.

Chikusetsu saponin IVa **53** (Figure 9), isolated from the Chinese herb *Aralia taibaiensis,* demonstrated a

cardioprotective effect by increasing SIRT1 activity, in agreement with previous studies highlighting that saponins from this plant exerted cytoprotective effects against oxidative stress induced by hyperglycemia [93]. In cardiomyocytes exposed to high glucose concentrations, an increased apoptosis rate and elevated ROS and calcium levels are the main signs of diabetic-induced myocardial injury. Pre-treatment of both H9c2 cells and neonatal primary cardiomyocytes with Chikusetsu saponin IVa reversed these effects, reducing the apoptotic population and mitochondrial ROS formation. These cardioprotective effects were ascribed to the ability of this saponin to increase SIRT1 expression. In *in vivo* studies, Chikusetsu saponin IVa protected myocardium from apoptosis, by increasing the levels of SIRT1 protein, thus demonstrating that this compound could be a promising agent for the treatment of diabetic cardiomyopathy [94].

Sulforaphane **54** (1-isothiocyanate-(*4R*)-(methylsulfinyl)butane, Figure 9) is a natural isothiocyanate present in cruciferous vegetables, which possesses well-documented cardioprotective properties [95, 96]. However, the exact mechanism of action of this compound has not yet been fully investigated. Sulforaphane showed a preventive effect on cardiomyocyte vitality during hypoxia/reoxygenation (H/R), by increasing cell viability. Moreover, it elevated the Bcl-2/Bax ratio, thus decreasing apoptosis, and it reversed the decreased mitochondrial membrane potential. The involvement of SIRT1 in these cardioprotective effects was established by measuring its expression in sulforaphane-pretreated cardiomyocytes, which resulted to be significantly increased, and the sulforaphane effects were partially abolished by a SIRT1 inhibitor, thus confirming that sulforaphane prevents H/R injury *via* activating SIRT1 signaling pathway [97].

Curcumin **55** (Figure 9), the active component of *Curcuma longa*, has a wide panel of biological activities and, among them, cardioprotection. Curcumin proved to be efficacious in increasing SIRT1 expression in both normal isolated rat hearts and cardiomyocytes; pretreatment with curcumin ameliorated the functional recovery of post-ischemic hearts, decreased infarct size and attenuated mitochondrial oxidative damage. The effect on SIRT1 expression was confirmed observing that a SIRT1 inhibitor and SIRT1 siRNA blocked the curcumin-mediated cardioprotective effects [98]. Recently, it was demonstrated that curcumin pretreatment alleviated hydrogen peroxide-induced endothelial premature senescence by reducing oxidative stress and apoptosis. These effects were due to both an increased SIRT1 expression and enzymatic activity by curcumin [99].

Melatonin **56** (Figure 9) is the main product secreted by the pineal gland and its cardioprotective action has been widely documented, with the involvement of many different signaling pathways [100]. Recent studies highlighted that melatonin pretreatment was able to protect against myocardial ischemia/reperfusion injury and to reduce the oxidative damage. Melatonin activates SIRT1 by increasing its expression and this effect is mediated by the melatonin receptor, since the cardioprotective effects of melatonin turn out to be antagonized by both a SIRT1 inhibitor and a melatonin receptor antagonist [101]. These effects were then confirmed *in vivo*, in a type 2 diabetic mouse model, in which melatonin improved cardiac functional recovery and decreased myocardial apoptosis, by up-regulating SIRT1 expression, which is instead attenuated in type 2 diabetic animals and further reduced after ischemia/reperfusion injury [102].

Glycoside rutin **57** (Figure 9), constituted by the flavonol quercetin and the disaccharide rutinose, can be found in some plants and vegetables such as passion flower, buckwheat, onions, apples and citrus fruits and it demonstrated potential beneficial effects for the treatment of osteoarthritis via SIRT1 activation. Rutin was found to increase the expression of SIRT1 in normal chondrocytes and to reverse the cytotoxicity and the inflammation induced by H_2O_2 treatment in hydrogen peroxide-treated chondrocytes; these effects were counteracted by SIRT1 inhibitor sirtinol, thus confirming the involvement of this enzyme in the mechanism of action of rutin. Treatment with rutin leads to the activation of SIRT1 with the consequent down-regulation of a series of pro-inflammatory factors and signaling pathways, which decreases the oxidative stress in rat chondrocytes [103].

Isorhamnetin **58** (Figure 9), the 3'-*O*-methyl derivative of quercetin, was able to increase the expression of SIRT1 when tested on cardiomyocytes, exerting cardioprotective effects against anoxia/reoxygenation-induced injury. Isorhamnetin increased cell viability, reduced ROS production, lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) release from cardiomyocytes, apoptosis and preserved the mitochondrial membrane potential. The involvement of SIRT1 was confirmed by the administration of sirtinol, which abolished the protective effects of isorhamnetin [104].

n-Tyrosol **59** [2-(4-hydroxyphenyl)ethanol, Figure 9] is a natural compound found in olive and argan oil and in white wine. Considering that resveratrol, present in red wine, was found to be able to activate SIRT1, an interest in investigating the possible action of *n*-tyrosol on SIRT1 led to studies to evaluate its cardioprotective effect. This natural compound was tested for myocardial ischemic stress in a rat *in vivo* model of myocardial infarction and pre-treatment with *n*-tyrosol proved to reduce both infarct size and cardiomyocyte apoptosis. The myocardial protection of *n*-tyrosol was ascribed to the ability of this phenolic derivative to induce the expression of SIRT1 [105].

The anthracycline antibiotic doxorubicin induces an oxidative stress that can contribute to the cardiotoxicity observed after its administration and, as a matter of fact, this side effect seriously limits its use as anticancer agent. In order to prevent this complication, natural lignin Sesamin **60** (Figure 9), present in sesame oil and seeds, proved to protect both H9C2 cardiomyocytes and animals from doxorubicin-induced cardiac injury. Sesamin up-regulates the protein expression of the antioxidant manganese superoxide dismutase enzyme Mn-SOD and also of SIRT1, an even more significant protein expression increase was observed in rats treated with the combination of doxorubicin and sesamin. Pretreatment with SIRT1 inhibitors partially reversed the cardioprotection exerted by sesamin on DOX-

induced cardiac damage, thus suggesting that Sesamin effects against cardiac toxicity should be at least partially mediated by SIRT1 activation [106].

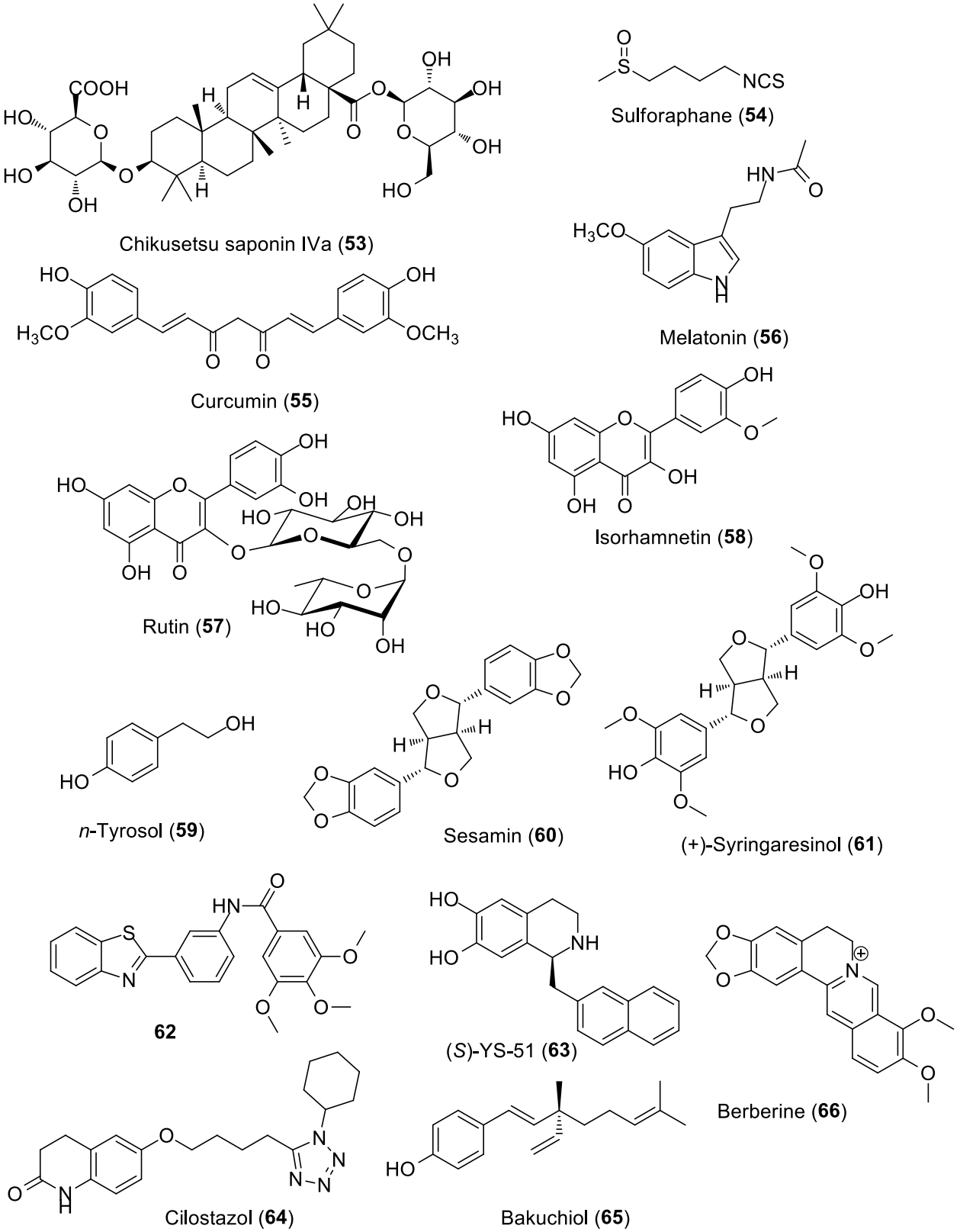

Figure 9. Structures of compounds increasing SIRT1 expression.

Structurally similar to Sesamin, syringaresinol **61** (Figure 9), isolated from *Panax ginseng* berry pulp, activates SIRT1 by promoting the binding of the transcription factor FOXO3 to a specific sequence of the SIRT1 promoter, leading to induction of SIRT1 expression in human umbilical endothelial HUVEC cells, as observed by the increased mRNA and protein level of SIRT1. This mechanism causes a delay of cellular senescence and leads to an improvement of endothelial functions [107].

N-(3-(benzo[*d*]thiazol-2-yl)phenyl)-3,4,5-trimethoxybenzamide **62** (Figure 9) represents one of the few examples of synthetic compounds that are able to increase SIRT1 mRNA expression (mRNA SIRT1/β-actin greater than 1.5). Moreover, it improves plasma and hepatic lipid profiles and decreases blood glucose concentration, thus demonstrating potential anti-diabetic properties [108].

The (*S*)-enantiomer of synthetic tetrahydroisoquinoline alkaloid YS-51 **63** (Figure 9) was found to be a potential therapeutic agent against the obesity-associated non-alcoholic fatty liver disease when tested in animals fed with high-fat diet that were supplemented with or without this compound. (*S*)-YS-51 turned out to improve the general lipid profile and insulin resistance of treated animals, and it reduced the inflammatory mediators in adipose tissue, as well as obesity, without affecting the food intake. These beneficial effects were correlated with the increased SIRT1 mRNA and protein levels both in the liver of high-fat diet mice and in HepG2cells, thus leading to the activation of AMPK pathway. Therefore, the protective effects of (*S*)-YS-51 are at least partially mediated by SIRT1 activation [109].

Cilostazol **64** (Figure 9) is a type III phosphodiesterase (PDE3) inhibitor that acts as a vasodilating antiplatelet drug to treat intermittent claudication. A protective effect of this compound on endothelial cells was observed, since cilostazol proved to be able to reduce vascular senescence. Cilostazol inhibited the premature senescence of endothelial cells induced by oxidative stress by increasing both SIRT1 mRNA and protein levels, as confirmed by the abrogation of this effect by treatment of HUVECs cells with a SIRT1 inhibitor. Cilostazol was effective also *in vivo*, since resection of the thoracic arteries of paraquat-treated mice (paraquat was administered to generate superoxide in the animals) highlighted an increase in SIRT1 expression and a decrease in the senescent morphological changes [110]. Moreover, cilostazol exerted positive effects also in neurodegenerative pathologies such as Alzheimer's disease by activating SIRT1 and related downstream target proteins [111, 112].

Bakuchiol **65** (Figure 9) is a meroterpene phenolic compound found in *Psoralea corylifolia* and in *Otholobium pubescens*. Bakuchiol exerts a protective role from IR-induced mitochondrial oxidative damage and this effect was found to be reversed by SIRT1 inhibitor Sirtinol and by SIRT1 siRNA, thus demonstrating an involvement of SIRT1 activation in its effect. An increased SIRT1 expression following bakuchiol treatment was observed both in normal and I/R-injured hearts and cardiomyocytes [113].

Another compound able to affect SIRT1 expression is Berberine **66** (Figure 9), a natural isoquinoline alkaloid which can be found in the Chinese herb *Rhizoma coptidis*, widely used in Chinese herbal medicine. Berberine showed a multitude of biological activities, and in particular it induced a significant increase of SIRT1 expression both *in vitro* and *in vivo*. This mechanism was considered to be responsible, at least in part, for the reduced myocardial damage against I/R injury observed after berberine treatment [114].

3. CONCLUSION

SIRT1 is implicated in many pathophysiological processes, such as metabolic diseases, inflammation, cancer, ageing diseases and cardiovascular pathologies, leading to a great interest toward this protein as a potential therapeutic target. In particular, the main positive effects elicited by SIRT1 activators in the cardiovascular system are summarized in Tables 3-5. However, both the activation and the inhibition of this enzyme can potentially cause positive effects, according to the involved pathways, and small molecules that behave as activators or inhibitors have so far been developed to opportunely modulate SIRT1. The first discovered SIRT1 activator was resveratrol, followed by other polyphenolic derivatives, but unfortunately their unspecific activity on additional different targets prevented their further development as drugs. Synthetic activators developed at Sirtris are among the most promising SIRT1 activators, since their selective modulation of SIRT1 to avoid any possible side effects deriving from modulation of other human sirtuins, together with a potent *in vitro* and *in vivo* activity, are the strictest requirements to develop potent SIRT1 activators. However, further studies to clearly elucidate the exact functions of SIRT1 in the cardiovascular system should be performed in order to investigate possible unwanted negative effects associated with chronic sirtuin activation. The class of "NAD^+ booster" compounds represent a new and debatable strategy for increasing SIRT1 activity, which aim at increasing the availability of the cofactor NAD^+. There is an unresolved debate about the safety of this strategy, since the alteration of the NAD^+:NADH ratio in the organism might provoke unpredictable consequences, due to the fact that NAD^+ is a cofactor involved in many crucial physiological processes.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Table 3. Beneficial cardiovascular effects elicited by natural SIRT1 activators.

Natural SIRT1 activators	Cardiovascular effects	Ref.
Resveratrol (**1**)	restores cardiac dysfunction in heart failure, reduces mortality, and improves cardiac function through the activation of SIRT1, thus increasing the expression of cardiac AMPK	[30]
	protects cardiomyocytes from oxidative stress, reducing ROS formation by a SIRT1-mediated mechanism	[32]
	promotes mitochondrial biogenesis in vitro and in vivo (endothelial cells and aortas of type 2 diabetic mice) through activation of SIRT1	[33]
	improves the cardioprotective effects of exercise training in aging rat hearts	[34]
	induces the expression of the transcription factor KLF2 in human vascular endothelial cells through SIRT1, leading to an endothelial vasoprotective effect	[35]
	SIRT1 activation ameliorates dystrophic cardiomyopathy by targeting cardiac p300 protein	[36]
	prevents cardiac hypertrophy, tissue fibrosis and preserves cardiac function, thus ameliorating cardiomyopathy in patients with muscular dystrophies	[37]
	protects coronary arterial endothelial cells against smoking-induced oxidative stress, by exerting antioxidant, anti-inflammatory, and antiapoptotic effects, mediated by SIRT1 activation	[38]
Pterostilbene (**7**)	attenuates ischemia-reperfusion injury in cardiomyocytes	[39]
Ginsenosides Rb_2 (**16**), Rc (**17**), F1 (**18**), Schisandrin A (**19**)	attenuate mitochondrial damage in cardiomyocytes in which oxidative stress was induced by *tert*-butyl hydroperoxide through activation of SIRT1, improving mitochondrial DNA content, oxygen consumption, and reducing ROS formation	[50]
β-Lapachone (**22)**	exerts cardioprotective effects in lipotoxic cardiomyopathy through SIRT1 and AMPK activation	[53]
Paeonol (**23)**	protects endothelial cells against hydrogen peroxide-induced premature senescence by modulating the expressions of SIRT1	[54]

Table 4. Beneficial cardiovascular effects elicited by synthetic SIRT1 activators.

Synthetic SIRT1 activators	Cardiovascular effects	Ref.
SRT1720 (**27**)	improves vascular endothelial function in old mice by enhancing COX-2 signaling and by reducing oxidative stress and inflammation	[65]
	protects against acute myocardial injury, especially in senescent and genetically engineered SIRT1-deficient hearts, by improving post-ischemic contractile function recovery and reducing infarct size	[66]
SRT2104 (**29**)	reduces arterial stiffness in healthy cigarette smokers and people with type 2 diabetes	[78]
Sildenafil (**42**)	exerts a protective effect against myocardial ischemia/reperfusion injury	[87]
1,4-Dihydropyridine-activators (**46-52**)	increases the mitochondrial function in murine myoblasts, with a mechanism involving PGC-1α activation by SIRT1	[91-92]
	high NO release in human keratinocyte cells	

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Table 5. Beneficial cardiovascular effects elicited by compounds increasing SIRT1 expression.

Compounds increasing SIRT1 expression	Cardiovascular effects	Ref.
Chikusetsu saponin IVa (**53**)	protects against hyperglycemia-induced myocardial injury in vivo and in vitro	[94]
Sulforaphane (**54**)	prevents myocardial hypoxia/reoxygenation injury in cardiomyocytes	[97]
Curcumin (**55**)	attenuates injury of post-ischemic hearts, decreases infarct size and decreases mitochondrial oxidative damage in both hearts and cardiomyocytes	[98]
	alleviates hydrogen peroxide-induced endothelial cells premature senescence by reducing oxidative stress and apoptosis	[99]
Melatonin (**56**)	attenuates myocardial ischemia/reperfusion injury by reducing oxidative stress damage in vivo	[101]
	ameliorates reperfusion-induced oxidative stress in a type 2 diabetic animal model	[102]
Isorhamnetin (**58**)	reduces anoxia/reoxygenation-induced injury in cardiomyocytes	[104]
n-Tyrosol (**59**)	induces myocardial protection against ischemia related stress in a in vivo model of myocardial infarction	[105]
Sesamin (**60**)	exerts cardioprotective effects in animal and cell models of Doxorubicin-induced cardiac injury	[106]

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