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Piia Kokkonen

Studying Sirtuin Inhibitors with In Silico and In Vitro Approaches

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PIIA KOKKONEN

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ABSTRACT

Caloric restriction (CR) is the kind of diet where the intake of calories is suppressed but the amount of nutrients is maintained; it has long been touted as the recipe for a longer lifespan. After the yeast enzyme silent information regulator 2 (Sir2) was found to be responsible for the longevity effects of CR, the search commenced to identify its analogues in mammals. Even though the roles of mammalian Sir2 relatives, sirtuins 1-7 (SIRT1-7), in CR and lifespan extension have not been definitely confirmed, sirtuins have become a major research target in health sciences. The function of sirtuins is linked to a wide variety of cellular functions, such as gene expression, energy production and adaptation to environmental stimuli.

Sirtuins form the class III of histone deacetylase enzymes (HDACs). They affect the function of their target proteins by mediating the deacetylation of lysine residues. During the last few years, some sirtuin subtypes have also been shown to possess other catalytic activities, such as demyristoylation and desuccinylation. All catalytic mechanisms of sirtuins involve the cofactor nicotinamide adenine dinucleotide (NAD^+). NAD^+ is a biomarker for cellular metabolism, and it is the utilization of this cofactor which places sirtuins in the crossroads of metabolism, gene expression and protein function.

The involvement of sirtuins in a wide variety of biological processes has triggered the search for sirtuin modulators, which could elucidate the physiological roles of sirtuins and ultimately function as sirtuin targeting drugs. Changes in sirtuin function have been proposed to affect several major pathological conditions including cancer, neurodegenerative diseases and diabetes. For example in these conditions it could be beneficial to inhibit sirtuin function, and thus effective sirtuin inhibitors have been sought for more than a decade.

In this thesis, modern computer aided drug design methods have been combined with *in vitro* methods in the investigation of the properties of sirtuin inhibitors. Often, sirtuin inhibitors are have been tested on SIRT1-3, whereas their effects on the lesser studied sirtuins i.e. SIRT4-7 have been ignored. Thus, as a starting point for the design of SIRT6 inhibitors, the effect of known sirtuin inhibitors on SIRT6 deacetylation was examined. In the subsequent screening for SIRT6 inhibitors, a new *in vitro* method was developed, based upon the natural deacetylation substrates of SIRT6. Then, data derived from interactions between substrate based inhibitors and SIRT1 was used in a quantitative structure-activity relationship study (QSAR) to design new inhibitors. Lastly, new small molecule sirtuin inhibitors were screened from a commercial database *in silico* to a putative allosteric binding site.

This thesis has resulted in the identification of new sirtuin inhibitors and novel insights into sirtuin inhibitor specificity. Additionally, the developed *in silico* and *in vitro* methods represent useful approaches for sirtuin inhibitor screening and drug design.

National Library of Medicine Classification: QU 26.5, QU 135, QU 143, QV 745
Medical Subject Headings: Caloric Restriction; Catalysis; Drug Design; Computational Biology; Histone Deacetylases; Inhibitors; NAD; Quantitative Structure-Activity Relationship; Sirtuins; In vitro Techniques

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TIIVISTELMÄ

Hiivan Sir2 (silent information regulator 2) entsyymin löytyminen ja sen toiminnan yhdistäminen kalorirajoitetun ruokavalion etuihin aloitti sitä vastaavien entsyymien etsinnän nisäkkäiltä. Kalorirajoitetussa ruokavaliossa huolehditaan ravintoaineiden saannista, mutta rajoitetaan ravinnon energiapitoisuutta, ja sitä on kauan pidetty elinikää pidentävänä ruokavaliona. Vaikka nisäkkäiden Sir2 entsyymien eli sirtuiinien 1-7 (SIRT1-7) yhteyttä kalorirajoitetun ruokavalion etuihin tai pidempään elinikään ei ole saatu varmistettua, ovat sirtuiinit nousseet tärkeäksi tutkimuskohteeksi terveystieteissä. Sirtuiinit vaikuttavat moniin solujen keskeisiin toimintoihin, kuten geenien ilmentymiseen, energiantuotantoon ja ympäristöön sopeutumiseen. Lisäksi muutokset sirtuiinien toiminnassa on yhdistetty useisiin sairauksiin, kuten syöpään, diabetekseen ja hermosolujen rappeutumissairauksiin.

Sirtuiinit ovat luokan III histonideasetylaaseja (HDAC). Deasetylaaseina ne katalysoivat asetyyliryhmien poistoa lysiineistä, mikä aiheuttaa muutoksia sirtuiinien kohdeproteiinien rakenteessa ja toiminnassa. Sirtuiinit käyttävät katalysaatiossaan nikotiiniamidi adeniini dinukleotidia (NAD^+), mikä tekee niistä ainutlaatuisia HDAC:ien joukossa, sillä muiden HDAC:ien katalysaatiomekanismi perustuu sinkkiin. NAD^+ on keskeinen solujen aineenvaihdunnan merkkiaine, ja sen käyttö laittaa sirtuiinit toimimaan aineenvaihdunnan, geenien ilmentymisen ja proteiinien toiminnan rajapinnalla. Muutaman viimeisen vuoden sisällä joillakin sirtuiineilla on havaittu myös muita katalysointimekanismeja, kuten demyristoylaatiota ja desukkinylaatiota. Sirtuiinien keskeinen rooli niin terveissä kuin sairaissakin soluissa on aloittanut sirtuiinien aktiivisuuteen vaikuttavien molekyylien etsinnän. Näillä yhdisteillä voidaan selvittää sirtuiinien fysiologista roolia, ja myöhemmin niitä voidaan käyttää sirtuiineihin vaikuttavien lääkeaineiden lähtökohtina.

Tässä väitöskirjassa seulottiin sirtuiinien inhibiittoreita yhdistämällä nykyaikaisia tietokoneavusteisen lääkeainesuunnittelun menetelmiä *in vitro* –tutkimusten kanssa. Aluksi tutkittiin tunnettujen sirtuiini-inhibiittorien vaikutusta SIRT6:n katalysoimaan deasetylaatioon. Usein sirtuiini-inhibiittoreiden vaikutus on tutkittu vain tunnetuimmilla sirtuiineilla SIRT1-3, kun vähemmän tutkitut SIRT4-7 on sivuutettu. Toiseksi uusien SIRT6-inhibiittoreiden seulomiseksi kehitettiin *in vitro*- menetelmä SIRT6:n luonnollisen substraatin pohjalta. Kolmanneksi substraatinkaltaisia inhibiittoreita käytettiin ensimmäistä kertaa kvantitatiivisena tiedonlähteenä suunniteltaessa uusia SIRT1-inhibiittoreita, joiden tehokkuus voitiin ennustaa *in silico*. Lopuksi kemialliselta rakenteeltaan uusia sirtuiinien inhibiittoreita seulottiin käyttämällä uutta mahdollista allosterista sitoutumisaluetta.

Väitöskirjatutkimuksen tuloksena löydettiin uusia sirtuiini-inhibiittoreita ja tehtiin havaintoja niiden alatyyppispesifisyydestä ja rakenne-aktiivisuussuhteista. Lisäksi käytetyt ja kehitetyt menetelmät todistettiin tehokkaiksi työkaluiksi sirtuiini-inhibiittorien seulonnassa, ja niitä voidaan hyödyntää myös tulevaisuudessa.

Luokitus: QU 26.5, QU 135, QU 143, QV 745
Yleinen suomalainen asiasanasto: aineenvaihdunta; entsyymit; inhibiittorit; lääkesuunnittelu; tietokoneavusteisuus; mallintaminen

Machines take me by surprise with great frequency.
-Alan Turing

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Kuopio, August, 2015

Piia Kokkonen

List of the original publications

This dissertation is based on the following original publications:

I Kokkonen P*, Rahnasto-Rilla M*, Kiviranta P, Huhtiniemi T, Laitinen T, Poso A, Jarho E and Lahtela-Kakkonen M: Peptides and Pseudopeptides as SIRT6 Deacetylation Inhibitors.
ACS Medicinal Chemistry Letters 12: 969-974, 2012.

II Kokkonen P*, Rahnasto-Rilla M*, Jarho E, Lahtela-Kakkonen M and Kokkola T: Studying SIRT6 Regulation Using H3K56 Based Substrate and Small Molecules.
European Journal of Pharmaceutical Sciences 63: 71-76, 2014.

III Kokkonen P, Mellini P, Nyrhilä O, Rahnasto-Rilla M, Suuronen T, Kiviranta P, Huhtiniemi T, Poso A, Jarho E and Lahtela-Kakkonen M: Quantitative Insights for the Design of Substrate-Based SIRT1 Inhibitors.
European Journal of Pharmaceutical Sciences 59: 12-19, 2014.

IV Kokkonen P, Kokkola T, Suuronen T, Poso A, Jarho E and Lahtela-Kakkonen M: Virtual Screening Approach of Sirtuin Inhibitors Results in Two New Scaffolds.
European Journal of Pharmaceutical Sciences 76: 27-32, 2015.

*The authors contributed equally.

The author's contribution in publications I-IV is as follows: in publication I, the author designed, conducted and reported the *in silico* studies of the inhibitors. In publication II, the author participated in designing of the *in vitro* method and designed, conducted and reported the *in vitro* studies of the inhibitors. In publication III, the author designed, conducted and reported the *in silico* studies and participated in the synthesis and purification of seven new inhibitors. In publication IV, the author participated in the designing of the *in silico* studies, analysed the results of the *in silico* screening and designed, conducted and reported the signal interference *in vitro* studies. The author substantially contributed to the composing, writing and editing of all the manuscripts.

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Abbreviations

Ac	acetyl
AceCS2	acetyl-coenzyme A synthetase 2
ADP	adenosine diphosphate
ADPR	adenosine diphosphate ribose
ALDH2	aldehyde dehydrogenase 2
AMC	7-amino-4-methylcoumarin
CADD	computer aided drug design
CoA	coenzyme A
CoMFA	comparative molecular field analysis
COPD	chronic obstructive pulmonary disease
CPS1	carbamoyl phosphate synthetase 1
CR	caloric restriction
GCN5	general control non-repressed protein 5
GDH	glutamate dehydrogenase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HIF1α	hypoxia-inducible factor 1-alpha
H	histone
HTS	high-throughput screening
HTVS	high-throughput virtual screening
K	lysine
IC_{50}	half maximal inhibitory concentration
IDH2	isocitrate dehydrogenase 2
KO	knock-out
LCAD	long-chain acyl-coenzyme A dehydrogenase
SOD1/2	superoxide dismutase 1/2
NAD^+	nicotinamide adenine dinucleotide
NAMPT	nicotinamide phosphoribosyltransferase
NF-κB	kappa-light-chain-enhancer of activated B cells
QM/MM	quantum and molecular mechanics
QSAR	quantitative structure-activity relationship
PDB	Protein Data Bank
PGC-1α	proliferator-activated receptor gamma coactivator 1α
PLS	partial least squares
PPAR	proliferator-activated receptor
ROS	reactive oxygen species
SD	standard deviation
SDH	succinate dehydrogenase
Sir2	silent information regulator 2
SIRT1-7	sirtuin 1-7
SNP	single nucleotide polymorphism
SOD1	superoxide dismutase 1
SP	standard precision
TCA	tricarboxylic acid cycle
TNF-α	tumour necrosis factor alpha
Tris-HCl	tris(hydroxymethyl)-aminomethane-hydrochloride
XP	extra precision

1 Introduction

Discovering and developing new drugs consumes huge amounts of time and resources. On average marketing a drug requires extensive interdisciplinary collaboration lasting many years with costs around 2.3 billion euros (DiMasi et al. 2014). As a way to streamline the complex process and to reduce the immense costs, computer aided drug discovery (CADD) has attracted popularity in the last decades. CADD can be used to limit the number of compounds needing to be examined in biological studies, to increase the hit rate of biological screenings and to optimize the pharmaceutical properties of new drug candidates. There are multiple examples where CADD has improved the hit rate of traditional high-throughput screening (HTS) or replicated the results of previous HTS campaigns with considerable less effort and cost (Sliwoski et al. 2013). The impact of CADD on drug design has been aided by the advances in computational software and hardware and the expanding knowledge of biological structures. The increasing structural and biological knowledge have also widened the field of possible drug targets to cover new and distinctive proteins outside the well-established targets, such as G-protein coupled receptors and kinases. A wide selection of new possible drug targets has emerged from the proteins involved in epigenetic regulation, which have expanded the gene expression beyond the DNA sequence.

All of the different cell types in the human body possess a general blueprint of all human proteins in their genome in the form of DNA. Although not modifying the genome itself, epigenetic changes alter the expression of proteins by silencing and activating genes. The delicate balance of gene silencing and activation makes it possible to have different functions and distinctive protein expression in different human cell types. The conversion of pluripotent stem cells into differentiated cells involves evident epigenetic changes, but epigenetics also affects cell adaptation to different environmental factors, such as the availability of nutrients. Epigenetic modifications can be made on the DNA itself, on various transcription factors or on the proteins around which DNA is wrapped, the histones. On histones, the epigenetic changes, such as methylation and acetylation, generally occur on the lysines in their flanking tail region. Acetylation of histone tail lysine residues is a unique post-translational modification because it neutralizes the positive charge of the lysine in physiological pH. This modification reduces the affinity of the histone tails towards the negatively charged DNA phosphate groups and thus the chromatin structure becomes looser and the genes in that region can become activated and translated into proteins. The delicate balance of histone tail acetylation level is controlled by two groups of enzymes. Histone acetyltransferases (HATs) transfer the acetyl (Ac) group to lysine residues, whereas histone deacetylases (HDACs) remove the Ac group. Even though it is known that acetylation affects a wide variety of proteins in addition to the histones, for historical reasons the enzymes controlling the phenomenon are still divided into these two categories (Peserico and Simone 2011).

HATs share a generally conserved catalytic mechanism, which involves acetyl-coenzyme-A (acetyl-CoA), whereas HDACs display more differences in their catalytic mechanisms. The so-called traditional HDACs in the phylogenetic classes I, II and IV utilize zinc in their deacetylation mechanism. Class III HDACs, or sirtuins, utilize nicotinamide adenine dinucleotide (NAD^+) as a cofactor while they undertake their catalytic activities. This connects sirtuin activity directly to the metabolic status of cells and means that sirtuins are unique drug targets among HDACs. Sirtuins function at the interface between the metabolism and gene expression, which has made them possible drug targets in major metabolic diseases, such as diabetes. Furthermore, the sirtuins also have key roles in neurodegeneration and tumorigenesis. However, there are still huge gaps in our understanding of sirtuins and their function.

Compounds which affect sirtuin activity either positively or negatively, could elucidate the role of sirtuins in both health and disease. For more than a decade, researchers have been looking for sirtuin activators and inhibitors has been facilitated by the increased structural knowledge and efficient *in vitro* methods (Villalba and Alcain 2012). Since computer models offer only approximations of the vastly complicated biological systems, in all cases CADD methods must be accompanied by experimental work. The main objective of this thesis was to utilize CADD methods together with *in vitro* methods to examine sirtuin inhibitors. In addition to providing new data of the recognized sirtuin inhibitors, multiple novel chemical entities were found to function as sirtuin inhibitors during the project. These novel sirtuin inhibitors can be used to further examine the physiological role of sirtuins and ultimately to serve as bases for drugs targeting these intriguing enzymes.

2 Review of the Literature

2.1 INTRODUCTION TO SIRTUINS

The founding member of the sirtuin family is the silent information regulator 2 (Sir2), which was a gene discovered in yeast *Saccharomyces cerevisiae*, which silences the mating type loci (Hicks et al. 1979; Ivy et al. 1985; Rine and Herskowitz 1987). Since then, one or more members of this highly conserved enzyme family have been found in virtually all species from archaea to mammals (Greiss and Gartner 2009). There are more sirtuin subtypes in complex organisms: only one sirtuin is present in bacteria, whereas most vertebrates have seven sirtuins (SIRT1-7) (Houtkooper et al. 2012). The function of sirtuins is dependent on the cellular metabolic marker NAD^+, which they use as a cofactor in the catalysis. Even though all sirtuins share a common catalytic domain and utilize the same co-substrate, they display different catalytic activities and substrate specificies (Nakagawa and Guarente 2011). All human sirtuins exhibit deacetylase activity on histones or other proteins *in vivo*. SIRT5 and SIRT6 can in addition deacylate longer than Ac fatty acyl modifications, and SIRT4 and SIRT6 display adenosine diphosphate (ADP)-ribosylation activity. Thus, by controlling the post-translational modification status of histones and other proteins, sirtuins can influence a wide variety of physiological and pathophysiological phenomena (Figure 1).

Sirtuins were discovered due to their gene silencing function, which is attributable mostly to their histone deacetylation activity (Sauve et al. 2001). In the deacetylation, sirtuins remove the Ac group from an acetylated lysine residue, restoring the positive charge on the lysine amino group (Figure 2). The reaction produces the deacetylated substrate, *O*-Ac-ADP-ribose and nicotinamide, which functions as a non-competitive sirtuin inhibitor. By deacetylating histone tails sirtuins increase the affinity of the histones for the negatively charged phosphate groups of DNA, which then bind the chromatin tighter around histones. This more tightly packed chromatin cannot be accessed by transcription machinery. Thus, the genes coded by that DNA region are silenced and cannot be translated into proteins. In addition to histones, sirtuins can deacetylate a wide variety of other proteins from transcription factors to enzymes, and each sirtuin subtype catalyses a set of slightly overlapping substrates (Rauh et al. 2013). The non-histone targets of sirtuins differ according to the sirtuin localization in cells (Figure 3): mitochondrial sirtuins have many targets in different stages of energy production, nuclear sirtuins contribute to DNA damage repair and in the cytosol, sirtuins affect various soluble proteins such as anti-oxidative enzymes.

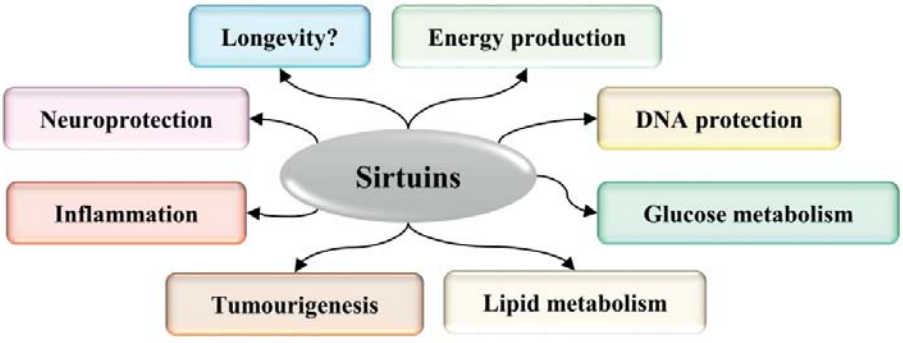


Figure 1. Examples of (patho)physiological phenomena, which have been connected to sirtuins.

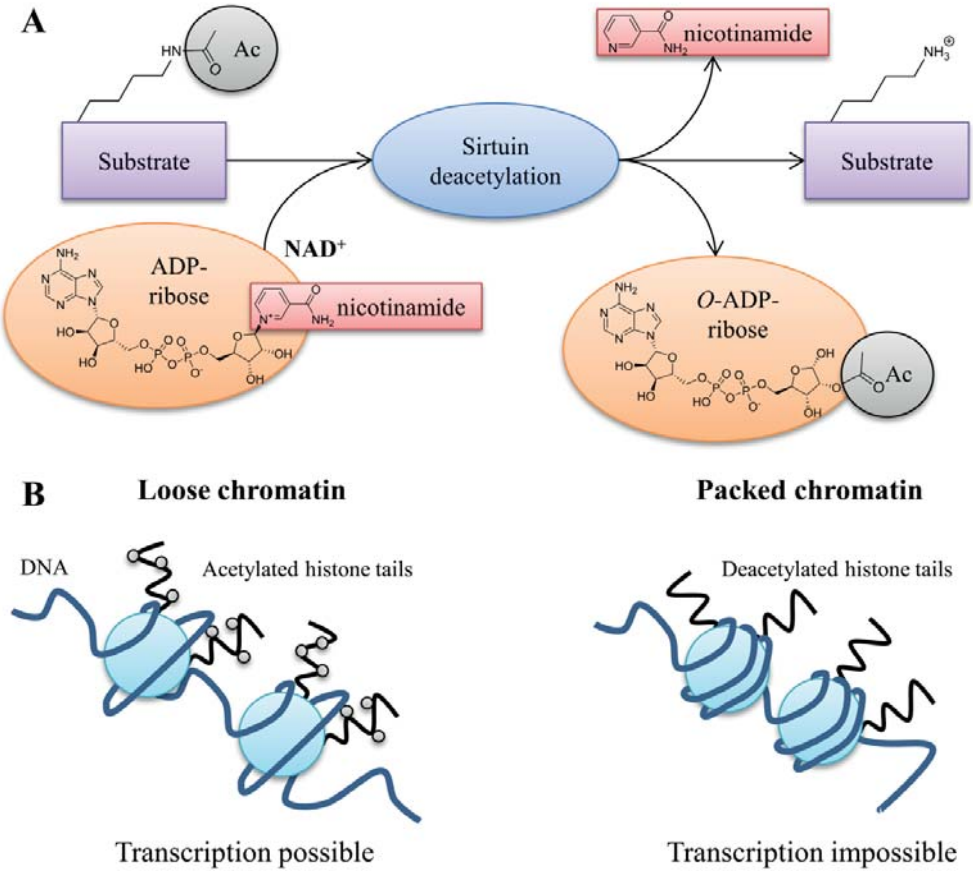


Figure 2. A. An overview of the deacetylation reaction catalysed by sirtuins. The reaction substrates are the protein substrate containing an Ac-lysine and NAD^+. The deacetylation reaction breaks NAD^+ to nicotinamide and *O*-ADP-ribose moieties, and the Ac group is transferred from the protein substrate to the *O*-ADP-ribose moiety. B. The simplified effect of histone tail acetylation status to chromatin structure and gene transcription.

The scientific interest towards sirtuins increased, when it was discovered that Sir2 was necessary for the caloric restriction (CR) mediated lifespan extension in yeasts (Aguilaniu et al. 2003). CR is a diet where the intake of calories is reduced 25-60% while the intake of nutrients such as vitamins and trace elements remains unchanged. It has long been known that CR can extend the lifespan of multiple organisms such as yeast, worms, fish, mice and rats (McCay et al. 1989; Weindruch et al. 1986; Weindruch et al. 1988). The lifespan of yeast, round worm and fruit fly could also be extended by increasing the expression of sirtuins, which boosted even further research into the properties of the sirtuins (Anderson et al. 2003; Lin et al. 2000; Lin et al. 2002; Rogina and Helfand 2004; Tissenbaum and Guarente 2001). Now, more than 10 years after the initial findings, there is still debate about what kind of role sirtuins play in CR and lifespan extension in mammals. Only a few sirtuin overexpressing mammal models have displayed extended lifespan (Colman et al. 2009; Kanfi et al. 2012; Naiman and Cohen 2012; Satoh et al. 2013). Interestingly, the effect of CR on the NAD^+ concentration and sirtuin activity depends on the tissue (Chen et al. 2008). In skeletal muscle, both the NAD^+ concentration and SIRT1 activity increase, but the opposite happens in liver. This is understandable, since in energy-deprived conditions sirtuins ensure the energy production in muscles, whereas the growth and metabolism controlled by liver can be delayed until more bountiful times. This also demonstrates that the

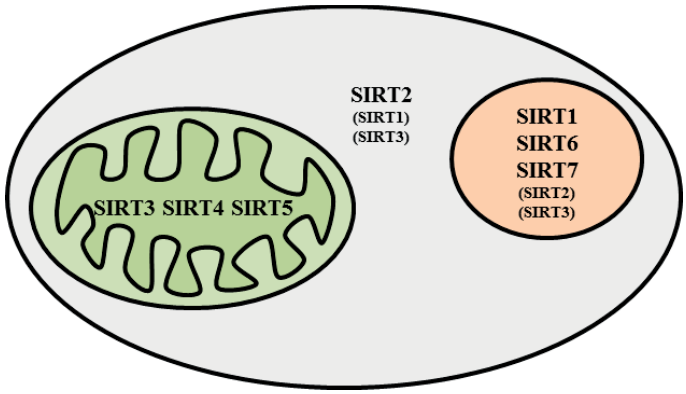


Figure 3. The general cellular localization of sirtuins. SIRT1, SIRT6 and SIRT7 reside mainly in the nucleus, but SIRT1 can shuttle to cytosol. SIRT2 is mainly cytosolic, but is transferred to the nucleus during mitosis. SIRT3 is present in all three cellular compartments: the full length SIRT3 resides in the nucleus and the cytoplasm. In the mitochondria SIRT3 is present as a short form, from which the mitochondria targeting part has been cleaved off.

deactivation or activation of sirtuins during CR is tissue-specific and thus it is much more complex than originally anticipated.

As demonstrated by CR, the NAD^+ dependent catalyzation mechanisms of sirtuins link their function to the metabolic status within the cells. NAD^+ is a key regulator of the cellular responses to external stimuli, such as nutrient availability (Imai 2009; Imai 2010; Imai and Yoshino 2013). A reduced nutrient input increases the biosynthesis of NAD^+ by the enzyme, nicotinamide phosphoribosyltransferase (NAMPT) (Revollo et al. 2004; Yang et al. 2007). An increase in the systemic NAD^+ concentration directly affects sirtuin activity in a tissue-dependent manner (Imai 2009; Imai 2010). These metabolic changes ensure the survival of cells in times of external perturbations. In addition to the many external stimuli which alter NAD^+ concentrations, the activity of the sirtuins has been shown to increase in response to internal types of stress, such as DNA damage and oxidative stress (Haigis and Sinclair 2010; Satoh et al. 2011). The fundamental role of sirtuins on stress survival is most clearly visible on cells with low levels of NAMPT, such as pancreatic β cells and neurons. These cells are extremely vulnerable to both intra- and extracellular stress, since their NAD^+ concentration and thus their sirtuin activities rely only on the extracellular sources of NAD^+ intermediates; nicotinamide mononucleotide and nicotinamide riboside. In mice, the supplementation of these intermediates has exerted beneficial effects via a sirtuin dependent mechanism on the metabolic abnormalities induced by high fat diet (Canto and Auwerx 2012). Even though in general the mitochondrial and cytosolic NAD^+ pools are considered to be independent on each other, the supplementation increased both the activities of the nuclear SIRT1 and the mitochondrial SIRT3 (Di Lisa and Ziegler 2001; Koch-Nolte et al. 2011).

2.2 MOLECULAR STRUCTURE AND SUBSTRATE BINDING

The information gathered from experimentally derived molecular structures is an indispensable part of the modern-day CADD. The sirtuin structures obtained from X-ray crystallographic studies provide valuable information about the catalytic mechanism, substrate specificity and how enzyme activity can be inhibited. Sirtuins contain a catalytic domain of around 275 amino acids, which is highly conserved from archaea and bacteria to mammals (Min et al. 2001; Yuan and Marmorstein 2012). The major structural differences between sirtuin subtypes lie mainly outside this conserved region, in the C- and N-terminals, which vary both structurally and sequentially. The N- and C-terminals contain sequences necessary for cellular localization,

regulation of the oligomerization state and auto-regulation mechanisms, and their structures remain unsolved possibly due to their flexible and unstructured nature (Schwer et al. 2002a; Tennen et al. 2010; Zhao et al. 2003). Nevertheless, several molecular structures have been published for the catalytic domains of human sirtuins (Table 1). A sequence alignment of sirtuin catalytic domains can be found in the review of Lavu et al. 2008.

The conserved catalytic domain of sirtuins consists of a large Rossmann fold domain and a smaller zinc-binding domain; these are connected by four linking loops (Figure 4). The substrate and cofactor binding site of sirtuins is located between the two domains. The zinc binding domain and the Rossmann fold form the general outline for the cleft and the linking loops participate to create the side walls. Sirtuins undergo a conformational change during cofactor and substrate binding. When there are no substrates present, sirtuins remain in a catalytically inactive open conformation (Figure 4) (Davenport et al. 2014). The binding of a protein substrate or NAD^+ induces a conformational change, which brings the two domains closer together. The major

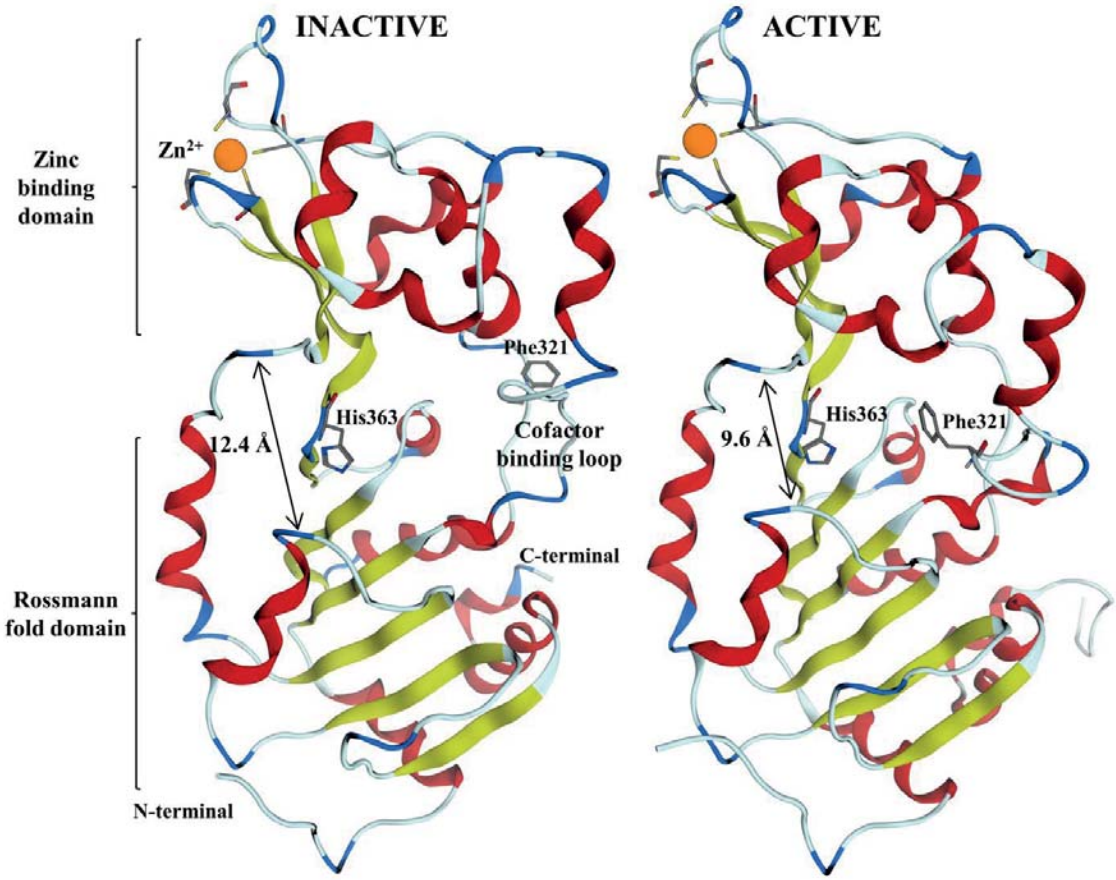


Figure 4. General structure of sirtuin catalytic domain in inactive and active conformations represented by SIRT1 crystal structures (PDB ID: 4IG9 and 4KXQ) (Davenport et al. 2014). The inactive conformation of sirtuins is observed in the absence of substrates. The substrates bind to a cleft between the larger Rossmann fold and the smaller zinc binding domains, where the catalytic histidine (His363) is located. The binding of substrates induces a conformational change, which brings the two domains closer to each other. The major conformational changes occur in the cofactor binding loop, which organises itself to form another half of the NAD^+ binding site. Colour coding for the ribbon: yellow = β-sheet, red = α-helix, blue and white = loop region. The structures were rendered with Molecular Operating Environment MOE v. 2014.3.

structural changes between the inactive and active conformations occur on a loop connecting the two domains; the cofactor binding loop (Figure 4). In the inactive conformation, the loop is disordered, but the binding of cofactor or substrate induces it to form the other half of the substrate binding site (Yuan and Marmorstein 2012). The reorganization of the loops also tilts the zinc binding domain closer to the Rossmann fold domain, and thus the substrate binding region becomes more closed.

The Rossmann fold domain is a characteristic feature of NAD^+ binding proteins (Rao and Rossmann 1973). It consists of a parallel β sheet consisting of six β strands surrounded on both sides by multiple α helices (Figure 4). While the structure of the Rossmann fold domain is conserved even beyond the sirtuin family, there are variations encountered in the structure of the zinc binding domain. It consists of a variable number of α helices and a β sheet consisting of three β strands held together by a zinc atom (Yuan and Marmorstein 2012). The zinc atom is held in place by four cysteine residues in a conserved sequence Cys-$X_{2–4}$-Cys-$X_{15–40}$-Cys-$X_{2–4}$-Cys (Sanders et al. 2010). The zinc atom is located too far from the active site to participate in the catalysis, and thus its function is assumed to be structural and necessary for the catalytic activity of sirtuins. Indeed, if the zinc atom is removed with a chelator, the structure of the small domain collapses and catalytic activity is lost (Chakrabarty and Balaram 2010). The catalytic activity can be restored with zinc supplementation.

The exact structures of the C- and N-terminals of sirtuins are not known, but they are expected to display disoriented structures without definitive α-helices or β-sheets (Liu and McCall 2013). Nonetheless, these terminals are essential for sirtuin function, since the removal of C- or N-terminals abolishes the deacetylation activity of SIRT1 (Pan et al. 2012). More precisely, the removal of N-terminal lowers catalytic rate, and the removal of C-terminal weakens the binding of NAD^+. The terminal segments contain structural and chemical features, which are involved in recognition and interaction with other proteins (Costantini et al. 2013).

Table 1. The published catalytic domain structures of human sirtuins, their resolution and co-crystallized molecules, such as substrates and inhibitors. PDB ID = the identification number of the protein structure in Protein Data Bank (PDB).

Sirtuin	PDB ID	Resolution (Å)	Bound molecules	Reference
SIRT1	4I5I	2.50	NAD^+, inhibitor	Zhao et al. 2013a
	4KXQ	1.85	ADPR	Davenport et al. 2014
	4IG9	2.64	-	Davenport et al. 2014
SIRT2	1J8F	1.70	-	Finnin et al. 2001
	3ZGO	1.63	-	Moniot et al. 2013
	3ZGV	2.27	ADPR	Moniot et al. 2013
	4L3O	2.52	macrocyclic inhibitor peptide	Yamagata et al. 2014
	4RMG	1.88	inhibitor, NAD^+	Rumpf et al. 2015
	4RMH	1.42	inhibitor	Rumpf et al. 2015
	4RMI	1.45	inhibitor	Rumpf et al. 2015
	4RMJ	1.87	ADPR, nicotinamide	Rumpf et al. 2015
	4R8M	2.10	peptide	Teng et al. 2015
SIRT3	3GLS	2.70	-	Jin et al. 2009

Table 1 continues on the next page.

Table 1. continued.

Sirtuin	PDB ID	Resolution (Å)	Bound molecules	Reference
SIRT3	3GLU	2.50	substrate peptide	Jin et al. 2009
	3GLR	1.80	substrate peptide	Jin et al. 2009
	3GLT	2.10	inhibitor peptide, reaction intermediate	Jin et al. 2009
	4FVT	2.47	substrate peptide, carba-NAD^+	Szczepankiewicz et al. 2012
	4JSR	1.70	inhibitor	Disch et al. 2013
	4JT8	2.26	inhibitor	Disch et al. 2013
	4JT9	2.24	inhibitor	Disch et al. 2013
	4BVF	2.05	inhibitor peptide, *O*-thio-Ac-ADPR	Gertz et al. 2013
	4BVH	1.90	inhibitor, *O*-Ac-ADPR	Gertz et al. 2013
	4BVB	2.00	inhibitor, ADPR	Gertz et al. 2013
	4BV3	2.00	inhibitor, NAD^+	Gertz et al. 2013
	4HD8	2.30	assay substrate peptide, piceatannol	Nguyen et al. 2013
	4BN5	3.25	inhibitor	Nguyen et al. 2013
	4BVG	2.50	substrate peptide, *O*-Ac-ADPR	Nguyen et al. 2013
	4C7B	2.10	assay substrate peptide, bromo-resveratrol	Nguyen et al. 2013
	4C78	2.00	assay substrate peptide, bromo-resveratrol	Nguyen et al. 2013
	4FZ3	2.10	assay substrate peptide	Wu et al. 2013
SIRT5	2NYR	2.06	inhibitor	Schuetz et al. 2007
	3RIG	2.00	substrate peptide	Du et al. 2011
	3RIY	1.55	substrate peptide	Du et al. 2011
	4F4U	2.00	substrate peptide, reaction intermediate	Zhou et al. 2012
	4F56	1.70	substrate peptide, reaction intermediate	Zhou et al. 2012
	4G1C	1.94	substrate peptide, carba-NAD	Szczepankiewicz et al. 2012
	4HDA	2.60	assay substrate peptide, resveratrol	Gertz et al. 2012
SIRT6	3PKI	2.04	ADPR	Pan et al. 2011
	3PKJ	2.12	*N*-Ac-ADPR	Pan et al. 2011
	3K35	2.00	-	Pan et al. 2011

2.2.1 NAD^+ binding site

The binding site of NAD^+ is represented in Figure 5 by the non-hydrolysable carba-NAD^+, which binds with a similar conformation (Du et al. 2011; Szczepankiewicz et al. 2012). For clarity, the SIRT3 numbering of residues is used both in Figure 5 and the text. The NAD^+ binding region is divided into three sites: site A for adenine binding, site B for nicotinamide-ribose binding and site C for nicotinamide moiety binding (Figure 5). Site A is mainly hydrophobic and shallow, and

offers many lipophilic contacts and dispersion interaction partners for the hydrophobic adenine moiety. In addition, there are conserved Asp365 and Asn344, which can form hydrogen bonds with the adenine and the adenine-ribose moiety, respectively (Yuan and Marmorstein 2012).

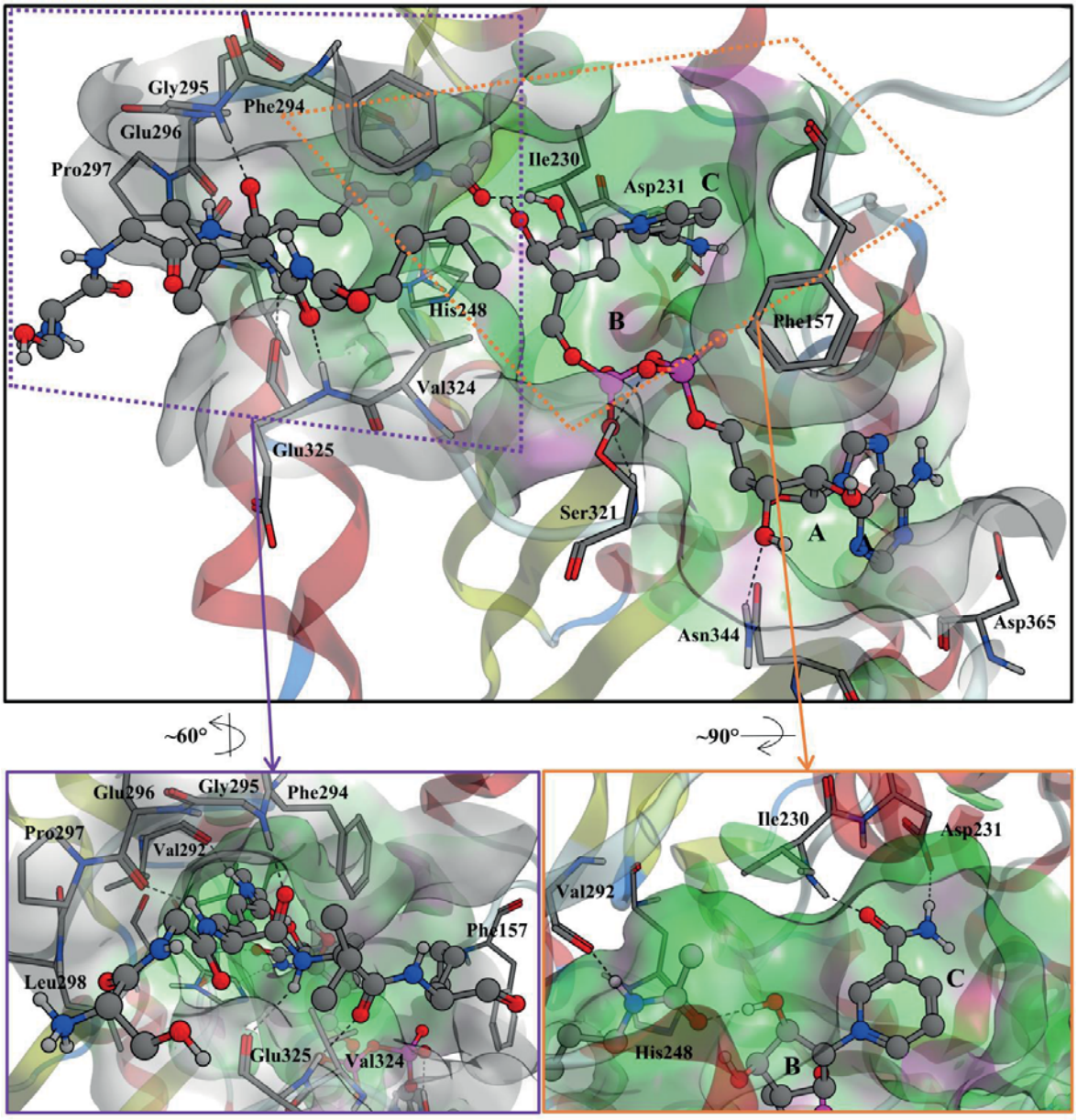


Figure 5. Sirtuin substrate binding site presented by SIRT3 crystal structure with bound substrate peptide and carba-NAD$^+$ (PDB ID: 4FVT) (Szczepankiewicz et al. 2012). Only the Ac-lysine of the substrate is inserted to the catalytic domain, whereas the rest of the substrate lies on the surface of the sirtuin. NAD$^+$ binding site is represented with the non-hydrolysable carba-NAD$^+$, and it is divided into three regions: site A for adenine, site B for nicotinamide-ribose and site C for nicotinamide binding. Colour coding for the ribbon: yellow = β-sheet, red = α-helix, blue and white = loop region. Colour coding for the substrate binding site surface: green = lipophilic, purple = polar, white = exposed to solvent. The structure was rendered with Molecular Operating Environment MOE v. 2014.3.

The pyrophosphate binding area between sites A and B contains multiple polar residues, such as the conserved Ser321, which form hydrogen bonds with the pyrophosphate groups (Figure 5). The nicotinamide-ribose moiety binds in close proximity to the catalytic histidine in the hydrophobic B site, where the hydroxyl groups of the ribose can form hydrogen bonds with the protein substrate. The nicotinamide moiety of NAD^+ binds to its site in the C-pocket, which provides hydrophobic contacts for the aromatic ring, and the carboxamide part of nicotinamide forms hydrogen bonds with two residues Ile230 and Asp231 (Figure 5) (Yuan and Marmorstein 2012). While Asp231 is conserved throughout the human sirtuins, Ile230 is replaced with valine in SIRT4 and SIRT6, and with cysteine in SIRT7 (Lavu et al. 2008).

The binding of NAD^+ affects the protein substrate conformation and thus its binding properties. The nicotinamide moiety of NAD^+ can bind to the B pocket instead of the C pocket. This type of NAD^+ binding is referred to unproductive, since the nicotinamide sterically prevents the protein substrate from reaching the catalytic histidine (Sanders et al. 2010).

It is unknown whether NAD^+ or the protein substrate binds first to induce the change from the inactive to active conformation. Initially it was thought that the protein substrate binds first, leading to NAD^+ binding (Cosgrove et al. 2006). Later, it was discovered that at least SIRT6 can bind NAD^+ in the absence of the protein substrate (Pan et al. 2011). The conformational changes of the cofactor binding loop during NAD^+ binding promote nicotinamide moiety to bind in the active conformation to the C-pocket. When NAD^+ is unbound and the sirtuin is in its inactive conformation, Phe157 (corresponding to Phe321 in Figure 4) of the cofactor binding loop resides in the C-pocket, thus preventing the productive binding of the nicotinamide moiety. During NAD^+ binding Phe157 moves to make space for the nicotinamide moiety and the cofactor binding loop forms the other half of the NAD^+ binding site. The residue corresponding to Phe157 is a similarly bulky and aromatic tyrosine in SIRT4 and SIRT7 (Lavu et al. 2008).

2.2.2 Lysine binding site

The structure of the lysine binding site of sirtuins is presented in Figure 5. For clarity, SIRT3 numbering is continued. Even though sirtuins catalyse the removal of many post-translational modifications, this chapter will focus on certain aspects of the N^{ε}-Ac-lysine binding. It is assumed that also the other post-translationally modified lysines bind to sirtuins in a similar fashion. For example, this can be seen in the crystal structure of succinyl-lysine bound to the SIRT5 (Du et al. 2011). However, it is noteworthy that the interactions discussed in this chapter depict only the interactions of the short Ac-lysine containing peptides crystallized together with sirtuin catalytic domains. The protein-protein interactions of sirtuins *in vivo* may involve contacts between other parts of the protein substrate and sirtuins.

The N^{ε}-Ac-lysine inserts itself into the lysine binding cavity to form hydrogen bonds with the nicotinamide-ribose moiety of NAD^+ (Figure 5). The N^{ε} of the Ac-lysine makes a hydrogen bond with Val292, which is conserved as a lipophilic residue between sirtuin subtypes, and the N^{ε} amide bond atoms make lipophilic contacts with the catalytic His248 (Sanders et al. 2010). The rest of the protein substrate chain lies outside the sirtuin, undertaking hydrogen bonding with the main chain atoms in residues 294-296 of the zinc binding domain and 324-325 in the Rossmann fold domain (Figure 5). This hydrogen bonding pattern resembles a staggered antiparallel β sheet and is referred to a β staple binding conformation.

In Figure 5 all of the hydrogen bonds of the protein substrate occur only with the main chain atoms of SIRT3. However, there are differences in sirtuin substrate specificities observed even with this kind of short peptides (Cosgrove et al. 2006; Kiviranta et al. 2009; Sanders et al. 2010). These differences arise from the side chains present on the protein substrate and the sirtuin subtype and possible interactions further away from the lysine binding area. In particular, the four to five residues on both sides of the Ac-lysine have been shown to affect sirtuin substrate specificity (Cosgrove et al. 2006). The backbone atoms of these residues make hydrogen bonds with the backbone amide hydrogens and carbonyl oxygens on both domains of the sirtuin as described above (Figure 5). However, the side chains of these residues could also be involved in

hydrogen bonding and dispersion interactions with the side chains of the sirtuin. In an acetylome peptide microarray study, the substrate specificities of human sirtuins were studied (Rauh et al. 2013). It was concluded that even though some of the sirtuin deacetylation substrate specificities overlap, the majority of the substrates remain unique to one particular sirtuin. The substrate specificity of sirtuins is controlled by the polarity of the residues close to the modified lysine. However, the microarray study did not rule out the effect of further residues on the substrate specificity, since only the closest residues were studied. Furthermore, sirtuins also displayed differences in deacetylation potencies; whereas SIRT1 deacetylated a broad range of substrates with lesser activity differences, SIRT2 displayed stronger deacetylation towards a smaller, more defined set of targets.

2.3 CATALYTIC MECHANISMS

The catalytic mechanisms through which sirtuins conduct their actions are not completely understood, even though multiple studies have been constructed around the topic (Avalos et al. 2004; Hirsch et al. 2011; Hirsch and Zheng 2011; Khan and Lewis 2006; Sauve and Schramm 2003; Sauve et al. 2006; Sauve 2010; Zhou et al. 2012). Since the deacetylation process is the pivotal sirtuin catalysed reaction, most of the studies have been focussed on it. It is generally agreed that the sirtuin catalysed NAD^+ dependent deacetylation occurs in two stages (Sauve 2010; Shi et al. 2013).

In the first reaction stage, the carbonyl oxygen of the Ac group undergoes a nucleophilic attack on the ribose moiety carbon bound to the nicotinamide moiety (Figure 6). This releases the nicotinamide and forms the peptidylimidate intermediate. The formation of this intermediate has not been directly confirmed, but the substrate mimicking inhibitors produce a corresponding stalled intermediate in many sirtuin X-ray structures (Jin et al. 2009). It is not entirely known if the initial nucleophilic attack follows an S_N1-like step-wise or an S_N2-like concerted mechanism. In S_N1-like mechanism, the glycosidic bond to nicotinamide is cleaved before the nucleophilic attack of the carbonyl oxygen, whereas these reactions occur simultaneously in the S_N2-like mechanism. In 2008 a quantum mechanics/molecular mechanics (QM/MM) study proposed the

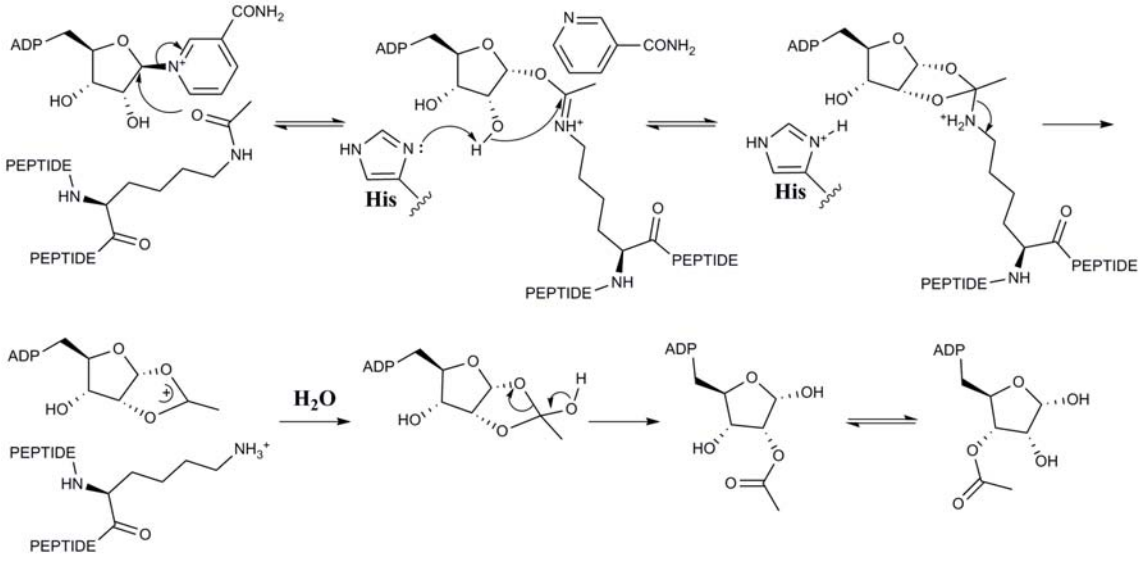


Figure 6. The sirtuin deacetylation reaction mechanism modified and combined from Sauve 2010 and Sauve and Youn 2012. The last step indicates the non-enzymatic spontaneous conversion between 2'-*O*-Ac-ADPR and 3'-*O*-Ac-ADPR.

reaction followed an S_N2-like mechanism, which was later supported by experimental kinetic studies and another QM/MM study (Cen and Sauve 2010; Hu et al. 2008; Liang et al. 2010). However, there is still no concensus if the mechanism is indeed S_N2, S_N1 or asynchronous S_N2, which involves features from both of these mechanisms (Sauve and Youn 2012).

The second reaction stage includes the rate-determining step and the contribution of the catalytic histidine (Min et al. 2001; Smith and Denu 2006). As described by Sauve, the second stage starts with the catalytic histidine acting as a general base to deprotonate one of the ribose hydroxyl groups, which leads to the formation of a bicyclic intermediate and the release of the deacetylated substrate (Figure 6) (Sauve 2010). Then, water decomposes the bicyclic intermediate to form the *O*-Ac-ADPR. This reaction order was questioned by a QM/MM study, which implied that the decomposition of the bicyclic intermediate occurred before the release of the deacetylated substrate (Shi et al. 2013). Whether these reactions take place in one order or another, there is experimental data that the two products of the second stage are released at the same time (Borra et al. 2004). In addition, both of the proposed reaction mechanisms have concluded that the participation of the catalytic histidine is the reaction limiting step (Sauve 2010; Sauve and Youn 2012; Shi et al. 2013).

The reaction mechanisms of the other sirtuin catalysed reactions have been less extensively studied. In addition to deacetylation, at least SIRT5 can catalyse the desuccinylation and demalonylation *in vitro* and *in vivo* (Du et al. 2011). It has been proposed that the reactions follow similar mechanisms as the deacetylation, since they produce corresponding *O*-succinyl- and *O*-malonyl-ADPR. ADP-ribosylation activity has been observed with SIRT4 and SIRT6 (Haigis et al. 2006; Liszt et al. 2005; Mao et al. 2011). The known substrates, glutamate dehydrogenase (GDH) and poly ADP-ribosylase 1 polymerase (PARP1) for SIRT4 and SIRT6, respectively, indicate that there is physiological role for this catalysis reaction (Haigis et al. 2006; Mao et al. 2011). Based on a bacterial sirtuin study, the mono-ADP-ribosylation activity of sirtuins occurs on the arginine or lysine two residues C-terminal from the Ac-lysine residue (Hawse and Wolberger 2009). The reaction starts initially in the same way as the deacetylation process, forming the ADPR-peptidylimidate intermediate. The nucleophilic arginine and lysine on the substrate protein can reach the intermediate from the surface of sirtuin and attack the ribose moiety. This leads to the formation of an ADP-ribosylated lysine or arginine, and the initially deacetylated lysine reverts back to being acetylated.

2.4 NUCLEAR SIRTUINS

The nucleus is the primary location for epigenetic control of gene expression, because it holds the majority of cellular DNA and the translation machinery. SIRT1, SIRT6 and SIRT7 reside mainly in the nucleus of cells and all of these sirtuins have deacetylation targets located in histone tails. Even though they share the same cellular location and NAD^+ pool, their deacetylation substrate specificities are different (Rauh et al. 2013). Thus, SIRT1 prefers mainly positively charged residues on both sides of the Ac-lysine, whereas SIRT6 prefers non-charged residues and SIRT7 favours hydrophilic non-charged or negatively charged residues. In addition to functioning as epigenetic gene silencers via histone deacetylation, SIRT1 and SIRT6 have multiple other deacetylation targets, and SIRT6 also has other enzymatic activities, including ADP-ribosylation activity. Nonetheless, the physiological function of SIRT7 and SIRT6 ADP-ribosylation and deacylation functions are not thoroughly understood.

2.4.1 SIRT1

SIRT1 has been the focus of human sirtuin research, since it is the closest homologue to the yeast life-prolonging Sir2 (Afshar and Murnane 1999; Smith et al. 2000). SIRT1 is a mainly nuclear sirtuin, which can shuttle to the cytoplasm (Byles et al. 2010; Hisahara et al. 2008; Sugino et al.

2010; Tanno et al. 2007). According to the Human Protein Atlas all normal tissue cell lines express SIRT1, mostly at high or medium levels. SIRT1 has robust deacetylation activity with more than 4000 identified deacetylation sites in more than 1800 proteins (Chen et al. 2012). SIRT1 has been called the master regulator of cellular metabolism in response to environmental changes (Holness et al. 2010). Since it can deacetylate various histone marks, transcription factors and other proteins SIRT1 affects many important cellular processes (Figure 7) (Hallows et al. 2006; Yang et al. 2006; Zhang and Kraus 2010). Despite being the closest homologue to the yeast life prolonging Sir2, the role of SIRT1 in CR and lifespan extension in mammals is still a matter of fierce debate. It has been shown that SIRT1 activity increases in some tissues during CR (Chen et al. 2008). However, the overexpression of SIRT1 does not lead to longer lifespan in mice, even though SIRT1 is involved in maintaining telomere length and promoting DNA repair (Palacios et al. 2010).

SIRT1 has an important role in both glucose and fatty acid metabolism especially in the liver. The first SIRT1 overexpressing mice exhibited a CR diet like phenotype: they were lean, active and displayed good blood lipid and glucose profiles (Bordone et al. 2007). Furthermore, two other SIRT1 overexpressing mouse strains did not show significant weight gain, glucose intolerance or insulin resistance even when fed a high fat diet (Banks et al. 2008; Pfluger et al. 2008). SIRT1 controls gluconeogenesis in the liver by increasing the expression of gluconeogenetic genes (Frescas et al. 2005). In addition to gluconeogenesis, SIRT1 also promotes the use of fatty acids in energy production in situations where glucose intake is insufficient. SIRT1 deacetylates peroxisome proliferator-activated receptor (PPAR) and PPAR gamma coactivator 1α (PGC-1α), which both promote fatty acid oxidation in the liver (Purushotham et al. 2009; Rodgers et al. 2005; Rodgers and Puigserver 2007). This is most notably seen in hepatic SIRT1 KO mice, which have dyslipidaemia and develop hepatic steatosis due to their dysfunctional lipid metabolism (Purushotham et al. 2009).

The level of SIRT1 expression is inversely correlated with inflammatory gene expression, but the causal effects are not known (Yoshizaki et al. 2009). This phenomenon can be observed in chronic obstructive pulmonary disease (COPD), where the presence of chronic inflammation decreases SIRT1 levels (Rajendrasozhan et al. 2008). The anti-inflammatory action of SIRT1 is supported by the direct deacetylation of p53 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Liu and McCall 2013; Yeung et al. 2004). This reduces inflammation and apoptosis triggered by environmental stimuli. In neurons, the anti-inflammatory action of SIRT1 is neuroprotective, and SIRT1 overexpression also reduces the formation of β amyloid plaques which are a hallmark of Alzheimer's diseases (Chen et al. 2005; Qin et al. 2006; Yeung et al. 2004). Interestingly, even though SIRT1 activity seems beneficial in some neurodegenerative diseases, the inhibition of SIRT1 has been reported to alleviate pathology in cell and mouse models of Huntington's (Smith et al. 2014). In that study, the beneficial results of SIRT1 KO could be duplicated with normal SIRT1 expression and treatment with a SIRT1 inhibitor. SIRT1 inhibition contributed to neuroprotection, but off-target effects could not be ruled out and the exact biological pathway was not clarified.

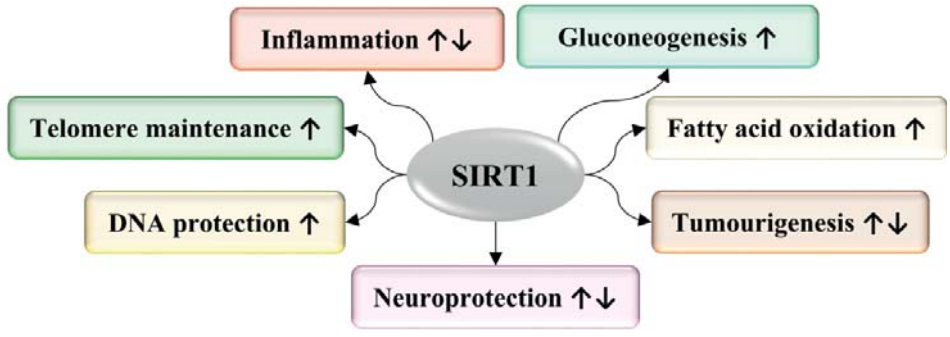


Figure 7. Examples of the biological functions mediated by SIRT1.

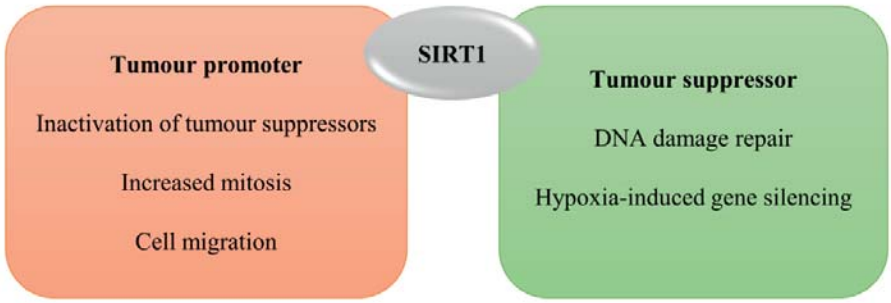


Figure 8. The tumour promoting and tumour suppressing functions of SIRT1.

SIRT1 has a dual role in cancer (Figure 8). SIRT1 is overexpressed in many human cancers; for example in leukaemia and prostate, breast, lung, colorectal, thyroid, gastric, hepatic, pancreatic and ovarian cancers (Feng et al. 2011; Herranz et al. 2013; Huffman et al. 2007; Jang et al. 2009; Kriegl et al. 2012; Lee et al. 2011; Noh et al. 2013; Wauters et al. 2013; Wu et al. 2012; Zhang and Wang 2013). In breast and prostate cancer, the extent of SIRT1 overexpression correlates with poor prognosis and metastasis (Lee et al. 2011; Lovaas et al. 2013; Nakane et al. 2012; Wu et al. 2012). Interestingly, the increase of SIRT1 expression in many cancer cell lines arises mostly from the increased cytoplasmic SIRT1 concentration, whereas the nuclear SIRT1 levels do not increase as extensively (Byles et al. 2010). Even though the function of cytoplasmic SIRT1 is predominantly unknown, SIRT1 has many deacetylation targets in the cytosol, for example, it can affect the mitotic activity of cancer cells and cell migration (Byles et al. 2010; Nakane et al. 2012). SIRT1 functions as a tumour promoter by inactivating or repressing the expression of tumour suppressors, such as p53 and retinoblastoma tumour suppressor protein (Vaziri et al. 2001; Wong and Weber 2007). However, SIRT1 has also tumour suppressive functions in some contexts. Some mouse strains with SIRT1 overexpression display a reduced susceptibility to intestinal tumour formation (Firestein et al. 2008). This might arise from SIRT1 controlling the cellular responses to hypoxia by silencing hypoxia induced genes, which can limit tumour growth (Lim et al. 2010). Generally, the DNA protective function of SIRT1 is tumour suppressive (Fan and Luo 2010; Ming et al. 2010; Yamamori et al. 2010). SIRT1 can maintain genome integrity in other ways, for example by recruiting repair factors to sites of DNA damage (Oberdoerffer et al. 2008; Wang et al. 2008). It seems that the contribution of SIRT1 to cancer is dependent on the context as well as on the aberrant pathways of specific cancers.

The activation of SIRT1 has been in the focus of research because of the metabolic benefits observed in SIRT1 over-expressing organisms. However, also SIRT1 inhibition offers interesting pharmacological possibilities. For example, pharmacologically-induced SIRT1 inhibition could be desirable in certain cancers and in some neurodegenerative diseases such as Huntington's.

2.4.2 SIRT6

SIRT6 has an important role as a cellular metabolism sensor and guardian of genomic stability, and it is expressed in all normal tissues (the Human Protein Atlas). An overview of the identified direct targets of SIRT6 and their function is presented in Figure 9. Initially, SIRT6 was identified as a nuclear mono-ADP-ribosylase with no detectable deacetylation activity (Liszt et al. 2005). It was also observed that SIRT6 has an auto-mono-ADP-ribosylation activity, although the physiological importance of this auto-regulation is still not clear (Etchegaray et al. 2013; Liszt et al. 2005). Later, poly-ADP-ribose polymerase 1 (PARP1) was discovered to be a direct SIRT6 mono-ADP-ribosylation target (Mao et al. 2011).

SIRT6 deacetylates lysines 9 and 56 on histone 3 (H3K9 and H3K56) (Kawahara et al. 2009; Michishita et al. 2008; Yang et al. 2009). This connects SIRT6 to chromatin and telomere maintenance, a property convincingly demonstrated in SIRT6 KO mice (Mostoslavsky et al. 2006).

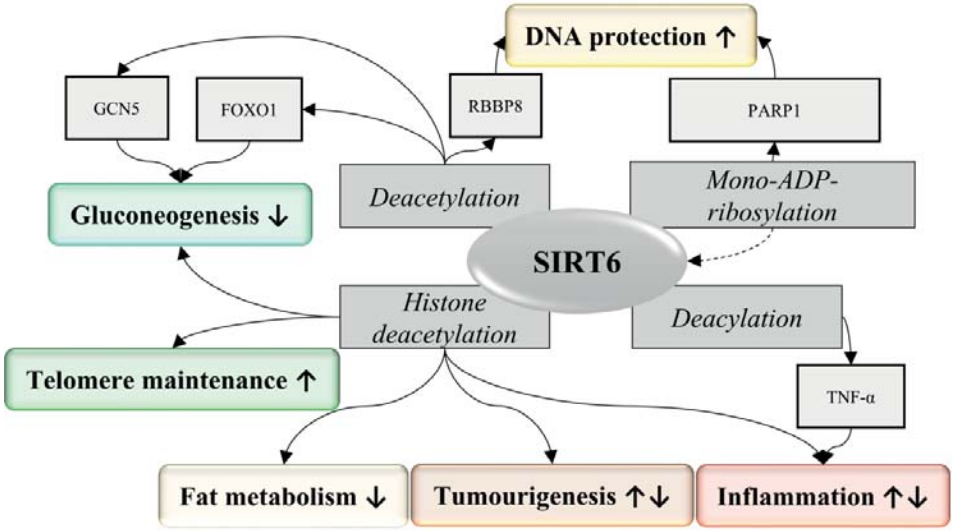


Figure 9. An overview of SIRT6 catalytic activities, identified targets and physiological functions. SIRT6 has been shown to catalyse auto-mono-ADP-ribosylation, but the effect of this function is not clear.

The mice developed symptoms related to ageing, such as loss of subcutaneous fat at the age of a few weeks. In addition, they had problems with their sugar metabolism, leading to severe hypoglycaemia, which caused them to die at around four weeks of age. The premature ageing observed in these mice was later demonstrated to be caused by the lack of telomere maintenance performed by SIRT6 (Michishita et al. 2008). The deacetylation of telomeric H3K9 and H3K56 increases the affinity of the chromatin towards Werner syndrome protein, which is an important genome stabilizer during DNA replication (Michishita et al. 2008). SIRT6 affects DNA repair mechanisms also through the deacetylation of DNA endonuclease RBBP8 and via mono-ADP-ribosylation of PARP1, which both promote DNA repair pathways (Kaidi et al. 2010; Mao et al. 2011).

SIRT6 is generally regarded as a tumour suppressor because of its DNA protecting function, and SIRT6 expression is known to be down-regulated in several human cancers (Lai et al. 2013; Sebastian et al. 2012). Increasing SIRT6 expression level in liver cancer initiation stage can impair tumour development (Min et al. 2012). Furthermore, cancer cells with SIRT6 deficiency display increased glycolysis and tumour growth (Sebastian et al. 2012). Controversially, SIRT6 has been reported to be overexpressed in chronic lymphocytic leukaemia (Wang et al. 2011a). SIRT6 levels were not down-regulated like those of other sirtuins in advanced states of lymphocytic leukaemia when compared to the early stages (Wang et al. 2011a). That study proposed that SIRT6 depletion was important for the initiation of the tumorigenesis, but later SIRT6 could protect the tumour from genetic instability.

SIRT6 expression has been shown to affect the lifespan of mammals (Kanfi et al. 2012). Male mice with SIRT6 overexpression lived 15% longer than their wild counterparts, but this phenomenon was not observed in females. The lifespan extension may be related to the tumour protective function of SIRT6. SIRT6 expression levels decline with age also in human pluripotent stem cells and an increase of expression of SIRT6 in these cells significantly increased their differentiation efficiency (Sharma et al. 2013).

As mentioned earlier, SIRT6 KO mice suffer from severe hypoglycaemia, which points to a key role for SIRT6 as a mediator of glucose metabolism (Mostoslavsky et al. 2006; Xiao et al. 2010). SIRT6 deficiency induces hypoglycaemia by increasing glucose uptake into cells (Xiao et al. 2010). SIRT6 also interacts with hypoxia-inducible factor 1-alpha (HIF1α) to repress mitochondrial

respiration and activate glycolysis (Zhong et al. 2010). Thus, when SIRT6 KO mice were treated with an HIF1α inhibitor, their hypoglycaemic phenotype was reversed. SIRT6 also reduces gluconeogenesis in the liver. SIRT6 directly deacetylates the general control non-repressed protein 5 (GCN5) enhancing its activity and suppressing hepatic gluconeogenesis (Dominy et al. 2012). SIRT6 also acts together with p53 and a transcription factor FOXO1 to diminish gluconeogenesis (Zhang et al. 2014).

In addition to sugar metabolism, also lipid metabolism is partially controlled by SIRT6. Mice with hepatic SIRT6 KO develop fatty liver disease because of enhanced glycolysis and triglyceride synthesis (Kim et al. 2010b). Furthermore, rosiglitazone treatment prevents lipid accumulation in the liver with a mechanism connected to SIRT6 expression (Yang et al. 2011). It has been claimed that visceral fat accumulation is controlled by SIRT6: mice with SIRT6 overexpression were protected against obesity caused by consumption of a high fat diet (Kanfi et al. 2012). The mice had significantly less visceral fat and their triglyceride and cholesterol levels were lower than those of their wild counterparts. SIRT6 overexpression lowers the blood cholesterol levels in diet-induced or genetically obese mice (Tao et al. 2013a; Tao et al. 2013b).

SIRT6 possesses a dual role in inflammation. On one hand, it acts in a pro-inflammatory manner through the deacylation of tumour necrosis factor alpha (TNF-α), which leads to its secretion from the macrophages (Jiang et al. 2013). On the other hand, it has been shown to deacetylate H3K9 at the promoter NF-κB controlled pro-inflammatory factors, including TNF-α (Kawahara et al. 2009; Xiao et al. 2012). This silences the transcription of the pro-inflammatory factors and thus reduces the intensity of the inflammation. These results may arise from the different cell types used in testing or they could imply that SIRT6 has different roles in inflammation depending on the cell type and environmental factors.

The inhibition of SIRT6 offers interesting prospects in the treatment of diabetes, because of the hypoglycaemia inducing effect of SIRT6 loss (Xiao et al. 2010). In addition, it has been proposed as a drug target in autoimmune diseases, because of its involvement in inflammatory processes (Bruzzone et al. 2009). It has been speculated that treatment with a SIRT6 inhibitor would probably benefit patients with SIRT6 overexpressing tumours, such as prostate cancer (Liu et al. 2013c).

2.4.3 SIRT7

The physiological relevance of SIRT7 has not been thoroughly studied, even though it is expressed in all normal tissues (the Human Protein Atlas). SIRT7 is expressed in higher levels in metabolically active tissues, such as liver and muscle. In cancer cells, SIRT7 is often overexpressed, which links this sirtuin to oncogenic transformation (Ford et al. 2006; Paredes et al. 2014). SIRT7 has been reported to be overexpressed in head, neck and hepatocellular carcinomas as well as thyroid and breast cancer, and it is expressed in almost all cancer cell lines in the Human Protein Atlas (Ashraf et al. 2006; Frye 2002; Kim et al. 2013; Lai et al. 2013). It has been proposed that SIRT7 can facilitate cell proliferation, by activatíng the transcription of ribosomal DNA by inducing RNA polymerase I transcription (Grob et al. 2009). SIRT7 also selectively deacetylates histone 3 lysine 18 (H3K18), which is an epigenetic biomarker of aggressive tumours and the hypoacetylation of H3K18 usually results in poor clinical outcomes in patients (Barber et al. 2012). The H3K18 deacetylation performed by SIRT7 leads to down-regulation of its target genes, some of which act as tumour suppressors. Further support for the concept that SIRT7 functions as an oncogene and tumour promoter, SIRT7 silencing RNA (siRNA) has been demonstrated to reduce the proliferation of hepatocellular carcinoma cells (Kim et al. 2013).

SIRT7 KO mice exhibit an ageing-like phenotype, have spinal deformations and degenerative cardiac hypertrophy at young age (Vakhrusheva et al. 2008). However, another strain of SIRT7 KO mice was resistant to high fat diet induced fatty liver, obesity, and glucose intolerance, even when the KO only occurred in the liver (Yoshizawa et al. 2014). Interestingly, these findings have been contradicted by two other groups, whose SIRT7 KO mice developed liver steatosis (Ryu et

al. 2014; Shin et al. 2013). Despite the controversy all studies have revealed that SIRT7 does play an important role in hepatic lipid metabolism and it has crucial roles in both cancer and normal cells, but because the details of its function are not known, more studies will be needed to elucidate the role of this enzyme.

2.5 MITOCHONDRIAL SIRTUINS

Mitochondria are the energy production organelles of cells, and their dysfunction is observed in a wide variety of pathophysiological conditions, such as cancer, diabetes and obesity. Sirtuins are the only enzyme family with deacetylase activity in the mitochondria (Michan and Sinclair 2007). However, not all acetylated mitochondrial proteins have been determined to be sirtuin substrates, leaving their acetylation status and its influence on physiological processes unclear. SIRT3 is the main deacetylase in mitochondria because the KO of SIRT3 increases the mitochondrial protein acetylation level significantly, whereas the KO of SIRT4 or SIRT5 has no effect (Lombard et al. 2007). All mitochondrial sirtuins, SIRT3, SIRT4 and SIRT5, exert catalytic activities on energy production related proteins, but they display different substrate specificities and enzymatic activities. SIRT3 catalyses only deacetylation, SIRT4 has specific deacetylation and ADP-ribosylation activities and SIRT5 can undertake deacetylation, demalonylation, deglutarylation and desuccinylation reactions. In addition, the deacetylation substrate preferencies for the mitochondrial sirtuins vary significantly: SIRT3 favours mainly positively charged residues around the Ac-lysine, SIRT5 favours negatively charged residues while SIRT4 does not exhibit as distinctive preferences as the other two sirtuins (Rauh et al. 2013).

2.5.1 SIRT3

SIRT3 is the main deacetylase present in the mitochondria (He et al. 2012b). Since undisturbed mitochondrial function is essential for cell vitality, it is not surprising that SIRT3 is expressed in all normal tissues. In a recent study, around 400 mitochondrial acetylation sites were found, and they were significantly hyperacetylated in the absence of SIRT3 (Hebert et al. 2013). SIRT3 is present in cells in two different forms: the full length and short. The full length SIRT3 is present in the cytosol and the nucleus, and it contains the mitochondrial targeting part for the intracellular transport of SIRT3 (Sundaresan et al. 2008). When SIRT3 has been imported into the mitochondria, the mitochondrial targeting part is cleaved off to produce the short form of SIRT3 (Schwer et al. 2002b). Both the full length and short forms of SIRT3 have deacetylation activity, in fact no other enzymatic activities have been discovered for SIRT3 (Lombard et al. 2007; Scher et al. 2007; Sundaresan et al. 2008). An overview of SIRT3 biological functions is presented in Figure 10.

SIRT3 is believed to be an important modulator of cardiac ageing and health, since SIRT3 KO mice suffer from early cardiac hypertrophy and fibrosis (Sundaresan et al. 2009). This is partially

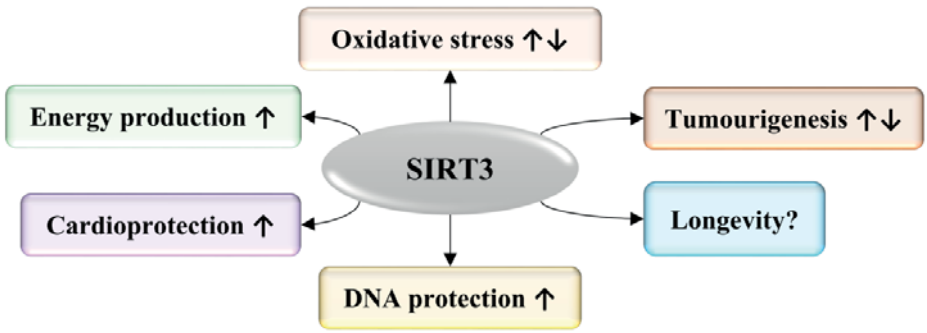


Figure 10. Examples of SIRT3 biological functions.

mediated by direct deacetylation of cardioprotective serine/threonine-protein kinase STK11 (Pillai et al. 2010). The cardioprotective function of SIRT3 is further supported by the deacetylation of several transcription factors, which increase the expression of antioxidant genes (Sundaresan et al. 2008). Furthermore, SIRT3 deacetylates and inhibits cyclophilin D (CypD), which opens the mitochondrial permeability transporter pore (Hafner et al. 2010). The opened pore induces mitochondrial swelling and dysfunction, eventually leading to cardiac hypertrophy.

Mitochondria are the main producer of reactive oxygen species (ROS), because some oxygen radicals are released from the electron transport chain (Finkel and Holbrook 2000). With ageing, the antioxidant systems of cells deteriorate and thus the amount of ROS in cells increases leading to oxidative damage of proteins, lipids and DNA (Rebrin and Sohal 2008). Neurons are especially sensitive to ROS accumulation, the outcome is a process called neurodgeneration (Shukla et al. 2011). SIRT3 directly acetylates many antioxidant enzymes, such as manganese superoxide dismutase 2 (SOD2) and isocitrate dehydrogenase 2 (IDH2) to increase their activity (Qiu et al. 2010; Someya et al. 2010; Tao et al. 2010; Yu et al. 2012). If the cells are deprived of glucose, a protein complex involving SIRT3 promotes the transcription of all mitochondrial DNA encoded genes (Peserico et al. 2013). Mice lacking SIRT3 display increased ROS levels and loss of mitochondrial DNA copies (Kim et al. 2010a).

In addition to controlling the ROS defences, SIRT3 is also important in regulating the mitochondrial energy and ROS production. During glucose deprivation and fasting amino acids and fatty acids are used for energy production. SIRT3 deacetylates GDH and IDH2 to ensure the supply of glutamate to the tricarboxylic acid cycle (TCA) during fasting (Schlicker et al. 2008; Verdin et al. 2010). SIRT3 also deacetylates targets in electron transport complexes I and II to maintain cellular energy production (Ahn et al. 2008; Finley et al. 2011b). Furthermore, during fasting acetate is released into the bloodstream to help with the energy demand of cardiac and skeletal muscles. In muscle cells, SIRT3 directly deacetylates and activates acetyl-CoA synthetase 2 (AceCS2), which creates acetyl-CoA from acetate, this can then be used as a cofactor in the TCA (Hallows et al. 2006; Schwer et al. 2006). SIRT3 also promotes the use of amino and fatty acids in energy production (Hallows et al. 2011; Hirschey et al. 2011). Remarkably, it has been shown that SIRT3 expression levels fluctuate depending on caloric input (Palacios et al. 2009; Tauriainen et al. 2011). If mice are fed a CR diet, their metabolically active tissues, such as liver, will display increased SIRT3 levels. In addition, exercise training increases SIRT3 expression levels in murine cardiac and muscle cells (Palacios et al. 2009). On the contrary, mice chronically fed a high fat diet exhibit suppressed SIRT3 activity and display hyperacetylation of mitochondrial proteins (Hirschey et al. 2011). This points to a role for SIRT3 in metabolic syndrome and the hyperacetylated mitochondrial proteins may also contribute to the liver injury induced by high fat diet (Choudhury et al. 2011).

SIRT3 affects tumorigenesis by controlling ROS levels, since almost all cancers display elevated ROS levels (Behrend et al. 2003; Liou and Storz 2010; Park et al. 2011). Furthermore, deletion of SIRT3 lowers the amount of activated oncogenes needed for tumour formation (Kim et al. 2010a). Generally it takes two oncogenic transformations to produce a tumour, but if there has been a SIRT3 deletion the amount is reduced to only one. HIF-1α stabilization has also been linked with SIRT3 tumorigenesis (Finley et al. 2011a). HIF-1α regulates the expression of genes in response to hypoxia, which is a common microenvironment in a tumour, and it has a widespread connection to functions involved in tumour formation, proliferation and the Warburg shift to mainly glycolytic metabolism (Semenza 2011a; Semenza 2011b; Vander Heiden et al. 2009; Warburg 1956).

All cancer cell lines of the Human Protein Atlas express SIRT3; generally the expression levels correspond to that found in healthy tissue. In oesophageal and colon cancers, patients with SIRT3 overexpression have generally poor outcome (Liu et al. 2014; Zhao et al. 2013b). SIRT3 expression also promotes the growth of lung cancer and oral squamous cell carcinoma (Kamarajan et al. 2012; Li et al. 2013). Controversially, in gastric cancer the expression of SIRT3 increases the survival rate of patients (Huang et al. 2014). Thus, it seems clear that the function of SIRT3 is

dependent on the cancer type and while it protects some tumours from ROS damage, the tumour suppressing functions of SIRT3 mean that its transcription has been silenced in some cancers. SIRT3 can also affect the efficacy of an anticancer agents, e.g. the levels of SIRT3 expression have been directly linked to tamoxifen resistance in breast cancer (Zhang et al. 2013). In that case increasing SIRT3 expression, the cells lose their sensitivity to tamoxifen and thus evade apoptosis.

Polymorphism of SIRT3 has been associated with a longer lifespan of humans (Bellizzi et al. 2005; Rose et al. 2003). In the male population of over 90 years of age, certain alleles of SIRT3 are non-existent indicating their detrimental effect on longevity (Bellizzi et al. 2005). A SIRT3 single nucleotide polymorphism (SNP) was claimed to increase survivability in the elderly (Rose et al. 2003). However, in 2009 this was questioned, since a new study found SIRT3 polymorphism important for longevity in certain, but not all, populations (Lescai et al. 2009). Longevity clearly depends on multiple genetic and environmental factors, and the role of SIRT3 SNPs in longevity needs clarification.

Similarly to the other sirtuins, the inhibition of SIRT3 offers possibilities in the therapy of various cancers, especially to counter the tamoxifen resistance in breast cancer (Zhang et al. 2013). Other suggested uses for SIRT3 inhibitors include the prevention of paracetamol-induced liver injuries and the treatment of ejaculation dysfunction (Lu et al. 2011; Mandava and Hellstrom 2013). SIRT3 prevents the toxicity of paracetamol by directly deacetylating aldehyde dehydrogenase 2 (ALDH2), which lowers the affinity of the toxic paracetamol metabolite for this enzyme (Lu et al. 2011). The hypothetical idea of SIRT3 inhibitors in the treatment of premature ejaculation arises from the fact that SIRT3 inhibition leads to decreased energy production in the smooth muscle cells, thus delaying the muscle contraction and ejaculation (Mandava and Hellstrom 2013).

2.5.2 SIRT4

The physiological role of SIRT4, like that of SIRT7, remains elusive. SIRT4 is expressed in medium or high levels in only ~20% of normal tissues (the Human Protein Atlas). Nevertheless, it is expressed to some degree in almost all cell types, with abundant expression in the placenta. Initially the mitochondrial SIRT4 was thought to be an ADP-ribosylase, as it displayed only very weak deacetylase activity *in vitro* (Haigis et al. 2006). SIRT4 ADP-ribosylates GDH and thus inhibits its activity (Haigis et al. 2006). In pancreatic β cells this leads to decreased amino acid dependent insulin secretion. Recently, it was discovered that SIRT4 possesses deacetylase activity and that it targets mitochondrial malonyl CoA decarboxylase (Laurent et al. 2013b). This leads to increased malonyl CoA concentration and lipogenesis.

SIRT4 KO cells display increased insulin sensitivity (Nasrin et al. 2010). Interestingly, this only happens when the cells are well nourished and CR seems to decrease the activity of SIRT4 even though the NAD^+ level increase. CR leads to increased glutamine metabolism and fatty acid β-oxidation via the loss of SIRT4 activity, but only if SIRT1 is present (Laurent et al. 2013a). The loss of SIRT4 also increased glutamine dependent proliferation of Burkitt lymphoma cells, whereas its overexpression sensitized the cells to energy deprived conditions (Jeong et al. 2013). SIRT4 KO mice display no definitive phenotype, but they are spontaneously prone to develop lung tumours (Jeong et al. 2013).

SIRT4 inhibition offers interesting possibilities in regulation of the activity or expression of other sirtuins. CR reduces the activity of SIRT4, whereas that of SIRT3 increases, but the causal effects of this dual activity are not clear (Laurent et al. 2013a). Furthermore, the KO of SIRT4 has been shown to increase SIRT1 expression, which implies that there is a connection between these two sirtuins (Nasrin et al. 2010). SIRT4 inhibitors could also be useful in treatment of type II diabetes because the loss of SIRT4 increases insulin sensitivity (Nasrin et al. 2010).

2.5.3 SIRT5

SIRT5 is expressed in medium to high levels in most of normal tissues and in 72% of cancer cell lines (the Human Protein Atlas). SIRT5 has many different enzymatic activities including

deacetylation, deglutarylation, demalonylation and desuccinylation (Du et al. 2011; Park et al. 2013; Tan et al. 2014). SIRT5 deacetylates carbamoyl phosphate synthetase 1 (CPS1) to upregulate the detoxification of ammonia originating from amino acids used in energy production (Du et al. 2011; Nakagawa and Guarente 2009; Schlicker et al. 2008). Recently, CPS1 was also confirmed as a deglutarylation target of SIRT5 (Tan et al. 2014). Both deglutarylation and deacetylation of CPS1 increase its activity and accelerate the urea cycle (Nakagawa and Guarente 2009; Tan et al. 2014). So far, no direct demalonylation targets of SIRT5 have been identified, even though several proteins have been determined to be malonylated (Du et al. 2011). Pyruvate dehydrogenase and succinate dehydrogenase complexes have been identified as SIRT5 desuccinylation targets (Park et al. 2013). Both of these complexes are part of the TCA cycle and by suppressing their biological activities, SIRT5 affects the mitochondrial respiratory chain. SIRT5 also desuccinylates superoxide dismutase 1 (SOD1) to reduce the mitochondrial ROS levels (Lin et al. 2013). The unsuccinylable mutant of SOD1 hinders lung tumour growth, which implies that SIRT5 may have a therapeutically exploitable role in tumour suppression.

The SIRT5 KO mice exhibit no metabolic abnormalities under basal conditions or when consuming a high fat diet (Yu et al. 2013). However, they show global hypersuccinylation of proteins and increased ammonia levels in blood during fasting. Interestingly, SIRT5 has been shown to protect cardiomyocytes from oxidative stress and SIRT5 promoter polymorphism has been linked to accelerated ageing in certain brain regions (Glorioso et al. 2011; Liu et al. 2013a). The physiological role of SIRT5 might be more important in times of stress than under normal conditions, but further studies will be needed to confirm this speculation.

2.6 CYTOSOLIC SIRT2

SIRT2 function is strongly connected to the cell cycle and differentiation. It is expressed in all tissues and in many cases, such as in central nervous system and in skeletal muscle, at high levels (the Human Protein Atlas). An overview of SIRT2 biological functions is shown in Figure 11. SIRT2 is a mainly cytosolic sirtuin, which was at first discovered to control the acetylation status of α-tubulin (Li et al. 2007; North et al. 2003). SIRT2 both deacetylases α-tubulin and interacts with HDAC6, which also acts as an α-tubulin deacetylase. The deacetylation of α-tubulin and transcription factors link SIRT2 to the regulation of cell differentiation, and this function has been demonstrated in adipocytes and oligodendrocytes (Jing et al. 2007; Li et al. 2007). During oxidative stress SIRT2 deacetylates and activates glucose-6-phosphate dehydrogenase (G6PD), which maintains the cellular levels of the antioxidant glutathione (Wang et al. 2014). This reveals a way for cells to adapt to environmental ROS stress within minutes by adopting a post-translational modification instead of reliance on the slower transcription based mechanisms.

Surprisingly for a cytosolic enzyme, SIRT2 deacetylases the nuclear histone 4 lysines 16 and 20 (H4K16 and H4K20), which directly regulate the chromatin structure and replication (Serrano et al. 2013; Vaquero et al. 2006). In the process of histone deacetylation, the normally cytosolic SIRT2 migrates to the nucleus before the start of mitosis (Inoue et al. 2007; North and Verdin 2007; Serrano et al. 2013; Wilson et al. 2006). The expression level of SIRT2 is also increased during mitosis, and its overexpression lengthens the duration of mitosis (Dryden et al. 2003). Because of its intrinsic role in cell division and differentiation, SIRT2 is considered to be a cancer related protein, and it is expressed in 12% of cancers, most abundantly in glioma (the Human Protein Atlas). SIRT2 has been reported to ensure the survival of glioma cells and to stabilize oncoprotein Myc, which is frequently over-expressed in cancers (He et al. 2012c; Liu et al. 2013b). However, similarly to other sirtuins, the role of SIRT2 in cancer is not simply tumour promoting. SIRT2 KO mice display no evident metabolic defects or phenotypes, but they develop different tumours depending on their gender, i.e. male SIRT2 KO mice have increased gastrointestinal tumour prevalence whereas the female develop more mammary tumours (Park et al. 2012). This suggests

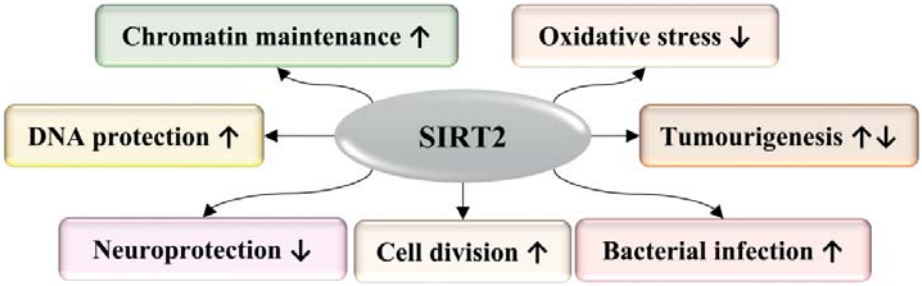


Figure 11. Examples of the biological functions modulated by SIRT2.

that the function of SIRT2 is tumour suppressive. Furthermore, the expression levels of SIRT2 are reduced in many cancers, such as hepatocellular carcinoma and breast cancer, providing more support for the tumour suppressing properties of SIRT2 (Kim et al. 2011).

Even though the exact mechanism of SIRT2 nuclear translocation remains unknown, SIRT2 is translocated to the nucleus also during bacterial infection (Eskandarian et al. 2013). By direct deacetylation of H3K18, SIRT2 induces the metabolic epigenetic changes imposed by the listeria pathogen and thus in SIRT2 KO cells, the bacterial infection was impaired. Interestingly, H3K18 is deacetylated by SIRT7 in context of cancer (see 2.4.3). H3K18 is a shared substrate for several sirtuins, being modified by different sirtuins under different conditions.

SIRT2 is abundantly expressed in the nervous system and its levels increase with age (Maxwell et al. 2011). This leads to an observable neurotoxic effect, which is connected with the enzymatic activity of SIRT2 and which has placed SIRT2 on the list of putative drug targets in neurodegenerative diseases (de Oliveira et al. 2012; Donmez and Outeiro 2013). SIRT2 inhibition has been shown to prevent the toxicity of α-synuclein aggregation in Parkinson's, but the exact mechanism of to explain this property remains unknown (Outeiro et al. 2007). SIRT2 inhibition was also suggested as beneficial for the treatment of Huntington's disease, but these results were later questioned (Bobrowska et al. 2012; Chopra et al. 2012). In addition to possibly preventing protein aggregation, SIRT2 controls neuronal autophagy, oxidative stress and inflammation, which all contribute to neurodegeneration (de Oliveira et al. 2012).

SIRT2 inhibition offers pharmacological possibilities for the treatment of bacterial infections and neurodegenerative diseases. The function of SIRT2 as well as that of the other sirtuins, and more generally HDACs, in neurodegeneration remains to intrigue scientists (Didonna and Opal 2015). Similar to the inhibitors of other sirtuins, also SIRT2 inhibitors could function as anticancer agents in certain malignancies, such as glioma (He et al. 2012c; Liu et al. 2013b).

2.7 SIRTUIN INHIBITORS

Classes I, II, and IV HDACs are validated anticancer targets. Many of their inhibitors are in clinical trials, and two of their inhibitors (vorinostat and romidepsin) have been approved by the FDA for the treatment of certain lymphomas. Although the function of sirtuins is similarly linked to fundamental metabolic processes, no sirtuin inhibitors are currently approved for clinical use and there is a continuous search for both the inhibitors and their clinical indications. Sirtuin inhibition could be useful in the treatment of different types of cancer, since sirtuins help the cancer cells to survive under stress. In addition, sirtuin inhibition has been proposed to help in neurodegenerative diseases, such as Parkinson's and Huntington's (Outeiro et al. 2007; Smith et al. 2014). Indeed, there is one sirtuin inhibitor undergoing the clinical trials and it is a drug candidate for Huntington's (for inhibitor details, see 2.7.3). Only reported inhibitors of human sirtuins will discussed here, even though the inhibition of bacterial and parasitic sirtuins could

have implications in the treatment of various infections (Chakrabarty and Balaram 2010; Religa and Waters 2012; Tavares et al. 2010; Tucker and Escalante-Semerena 2010).

2.7.1 Physiological inhibition

Generally, the products of enzyme catalysed reactions can be viewed as starting points for inhibitor design (Wolfenden 1999). The nicotinamide produced by the deacetylation reaction of sirtuins is known to function as the physiological inhibitor of sirtuins (Figure 12) (Bitterman et al. 2002). The inhibition mechanism of nicotinamide lies in its capability to rebind to the sirtuin and perform a nucleophilic attack on the peptidylimidate intermediate (Sauve et al. 2001; Sauve and Schramm 2003). This prevents the progression of the deacetylation reaction and converts the intermediate back to the initial reactants. The reported half maximal inhibitory concentrations (IC_{50}) of nicotinamide with different sirtuins are generally in the low micromolar level in the range of 30-200 μM (Grozinger et al. 2001; Suzuki et al. 2006; Yang and Sauve 2006). The cellular nicotinamide concentration is around 100 μM, which implies that the nicotinamide concentration in cells is high enough to regulate sirtuin activity (Sauve 2010; Schemies et al. 2010).

Nicotinamide is the amide of nicotinic acid, also known as vitamin B3, and these two compounds can be converted into each other *in vivo* (Belenky et al. 2007). The depletion of nicotinamide can lead to a condition called pellagra; its symptoms include skin lesions and dementia, but this condition is generally avoidable by normal food intake (Hegyi et al. 2004). Nicotinamide is an approved nutritional supplement and it is in phase II clinical trials for the treatment of polycystic kidney disease (clinical trial NCT02140814), and it is possible that SIRT1 inhibition contributes to this effect (Mellini et al. 2015; Zhou et al. 2013).

Naturally, the physiological inhibitor of sirtuins has intrigued researchers who have attempted to develop more potent nicotinamide analogues. Generally it has been difficult to improve the inhibition potency with synthetic modifications of the nicotinamide scaffold (Jackson et al. 2003; Schmidt et al. 2004). However, a 2-anilinobenzamide which has an IC_{50} value of 17 μM against SIRT1 is a somewhat more potent inhibitor than nicotinamide itself (Figure 12) (Suzuki et al. 2006).

2.7.2 Substrate based inhibitors

In addition to reaction products, modified substrates are another typical starting point for inhibitor design (Wolfenden 1999). When developing substrate based inhibitors of sirtuins, the analogues of NAD^+ are generally not favoured due to two reasons. Firstly, NAD^+ is a cofactor or a cosubstrate in a multitude of biochemical reactions, and therefore it might be difficult to achieve sirtuin specificity with some analogues. Secondly, NAD^+ possesses a charge at physiological pH, which could lead to problems for the analogues in terms of their in cell permeabilities. Nevertheless, some inhibitors have been developed based on NAD^+. The non-hydrolysable carba-NAD^+, which is present in some sirtuin crystal structures, is a weak inhibitor of sirtuins (Figure 13) (Landry et al. 2000). It hinders the nicotinamide cleavage and the subsequent formation of the reaction intermediate.

The adenosine moiety of NAD^+ inspired a research group to test adenosine based kinase inhibitors for their sirtuin inhibitory properties (Trapp et al. 2006). The most potent inhibitor

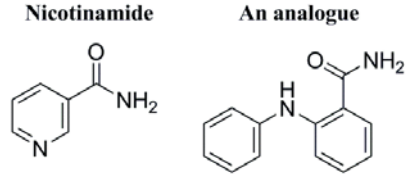


Figure 12. Physiological sirtuin inhibitor nicotinamide and 2-anilinobenzamide, which is an example of its potent analogues.

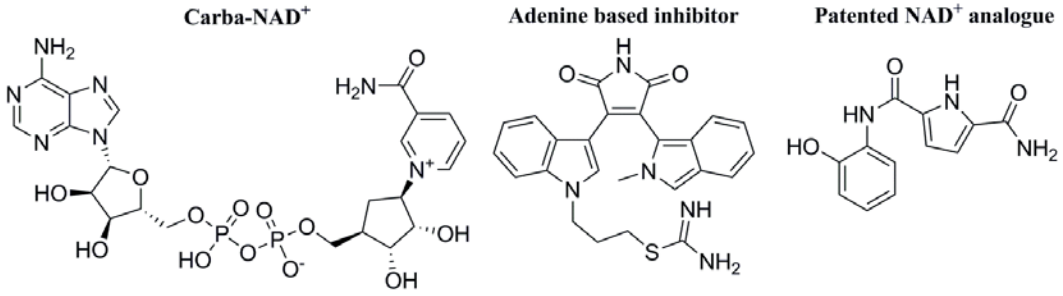


Figure 13. Examples of sirtuin inhibitors based on NAD^+.

found from this series had low micromolar IC_{50} values of 4 μM and 1 μM for SIRT1 and SIRT2, respectively (Figure 13). Furthermore, a compound has been patented as an analogue of the nicotinamide moiety of NAD^+ (Figure 13) (Mellini et al. 2015). This compound exhibited a low micromolar 1 μM IC_{50} value against SIRT1 in an *in vitro* assay.

Because of the aforementioned problems with the analogues of NAD^+, the analogues of N^ε-Ac-lysine in short peptides have become the focus of the substrate based sirtuin inhibitor research. The first inhibitor developed in this series is the thio-Ac-lysine containing peptide discovered by Fatkins et al. in 2006. Since then, a myriad of N^ε-Ac-lysine analogue containing inhibitors or experimental substrates have been published (Figure 14). They include tens of N^ε-thio-Ac-lysine containing inhibitors together with N^ε-thiocarbamoyllysine, N^ε-isothiovaleryllysine, N^ε-isovaleryllysine, N^ε-3,3-dimethylacryllysine and N^ε-trifluoro-Ac-lysine containing inhibitors (Figure 14) (Hirsch and Zheng 2011; Hirsch et al. 2011; Huhtiniemi et al. 2010; Kiviranta et al. 2009; Smith and Denu 2007a).

N^ε-thio-Ac-lysine containing peptides are recognized by sirtuins as substrates and they go through the dethioacetylation reaction, undergoing a similar reaction mechanism as the deacetylation process (Fatkins and Zheng 2008; Jin et al. 2009; Smith and Denu 2007a; Smith and Denu 2007b). The first reaction step functions similarly as in the deacetylation and nicotinamide

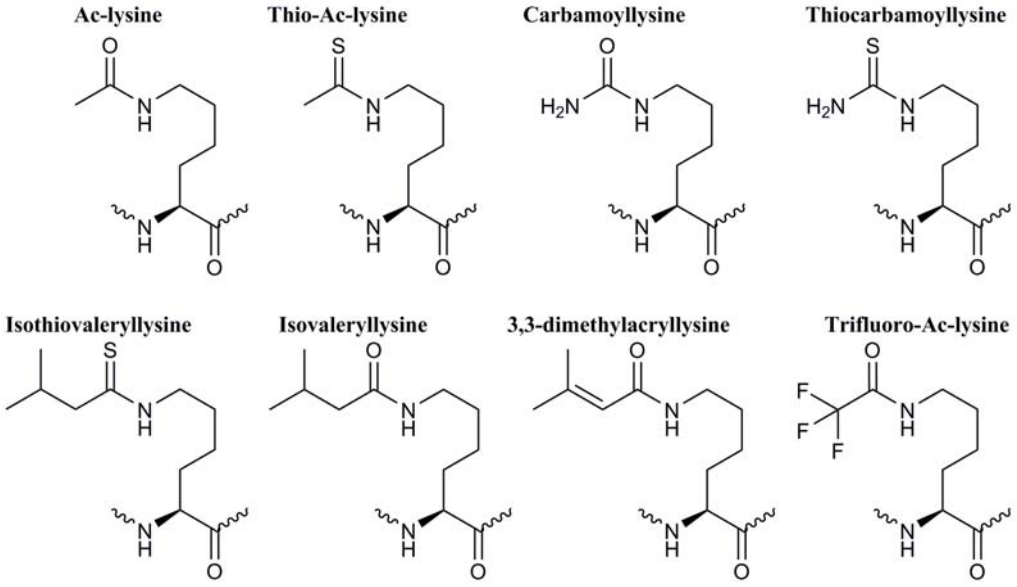


Figure 14. N^ε-Ac-lysine and examples of its inhibitory analogues, which have been included in different peptide sequences.

is released. The *S*-alkylamidate peptidyl intermediate stalls the reaction by hindering the hydroxyl attack to the acetyl carbon and the subsequent formation of the bicyclic intermediate. This is due to the *S*-alkylamidate carbon being a weaker electrophile than its *O*-alkylamidate counterpart; in addition, the *S*-alkylamidate has non-ideal bond lengths and angles for the nucleophilic attack. The thio-Ac-lysine thus acts as a tight-binding stalled intermediate inhibitor. The first thio-Ac-lysine containing sirtuin inhibitor was a peptide based on p53 residues 372–389 (Fatkins et al. 2006). Since then, many natural peptide sequences with thio-Ac-lysine have been published (Fatkins and Zheng 2008; Kiviranta et al. 2009; Smith and Denu 2007b). The most potent thio-Ac-lysine containing compounds display inhibitory activities in the sub-micromolar range. One example of these is the SIRT3 substrate AceCS2 residues 633–652 based inhibitor with a thio-Ac-lysine, which has a SIRT1 IC_{50} value of 0.9 μM (Fatkins and Zheng 2008).

N^{ε}-carbamoyllysine and N^{ε}-trifluoro-Ac-lysine containing inhibitory substrates developed soon after discovery of the inhibitory properties of the N^{ε}-thio-Ac-lysine moiety (Khan and Lewis 2006; Smith and Denu 2007b; Smith and Denu 2007c). They were included in sequences based on residues 9-19 of H3. N^{ε}-trifluoro-Ac-lysine contains an electron-withdrawing substituent, which decreases the nucleophilicity of the Ac oxygen (Smith and Denu 2007c). This results in a reduced catalyzation rate, as it slows down the rate-determining step by forming a stalled intermediate, similarly as occurs with the N^{ε}-thio-Ac-lysine containing inhibitors. N^{ε}-carbamoyllysine goes through the catalysed reaction, but no corresponding *O*-ADPR-analogue is released (Khan and Lewis 2006). The authors propose this is due to water regenerating the reaction intermediate back to the inhibitor, N^{ε}-carbamoyllysine. This inhibition mechanism inspired another group to develop an N^{ε}-thiocarbamoyllysine containing peptide (Hirsch et al. 2011). It forms a more stable intermediate and thus has better inhibition potency than the carbamoyllysine peptide. The SIRT1 IC_{50} value of the developed N^{ε}-thiocarbamoyllysine containing peptide was 6 μM.

N^{ε}-isothiovaleryllysine, N^{ε}-isovaleryllysine, N^{ε}-3,3-dimethylacryllysine and N^{ε}-seleno-Ac-lysine containing peptides were developed in order to study the dimensions of the SIRT1 and SIRT2 substrate binding sites (Huhtiniemi et al. 2010). The inhibitory N^{ε}-Ac-lysine analogues were included into sequences of Ac-Ala-Lys(modified)-Ala-OH. The IC_{50} values of N^{ε}-isovaleryllysine and N^{ε}-3,3-dimethylacryllysine containing inhibitors were in the low micromolar range. Interestingly, N^{ε}-seleno-Ac-lysine and N^{ε}-isothiovaleryllysine containing peptides displayed inhibitory activity comparable to that of their N^{ε}-thio-Ac-lysine counterparts. Their SIRT1 IC_{50} values were 0.4 μM and 0.7 μM, respectively. The inhibition mechanisms of these compounds have not been elucidated.

Since especially SIRT5 and SIRT6 can catalyse longer lysine modifications than Ac, their inhibitors have also been developed also from those substrates (Du et al. 2011; Jiang et al. 2013; Park et al. 2013; Tan et al. 2014). The desire to achieve a longer lived intermediate formation, which would be similar to that of the N^{ε}-thio-Ac-lysine containing inhibitors, was the driving force in the design of thiosuccinyllysine containing peptide as a SIRT5 and thiomyristoyllysine containing peptide as a SIRT6 inhibitor (Figure 15) (He et al. 2012a; He et al. 2014). The inhibitors were designed on the H3K9 peptide sequence and they exhibited low micromolar activities

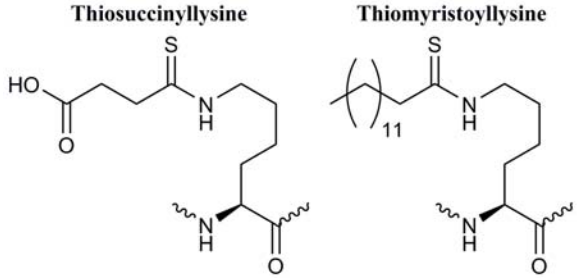


Figure 15. Inhibitory N^{ε}-modifications, which have been included in H3 peptide sequences.

Figure 16. Examples of potent pseudopeptidic sirtuin inhibitors.

against their target sirtuins. Interestingly, even though the corresponding substrates are specific for these sirtuins, the inhibitors display differences in specificity. Whereas the thiosuccinyllysine peptide shows more than two-fold specificity towards SIRT5, thiomyristoyllysine inhibitor displays equipotent activity between SIRT1-3 and SIRT6 (He et al. 2012a; He et al. 2014).

Even though some of the substrate based inhibitors possess good subtype specificity and potency, their testing in cells and further development to drugs is difficult because of the general problems of peptides (Fosgerau and Hoffmann 2014). Typically peptides are not orally bioavailable, and they display poor cell penetration and inadequate metabolic stability. To combat these issues, some inhibitory lysine analogues have been combined with synthetic chemical structures instead of natural amino acids to produce pseudopeptides (Huhtiniemi et al. 2011; Mellini et al. 2013; Suzuki et al. 2009). One set of potent pseudopeptides from different studies is shown in Figure 16 and they display IC_{50} values of 2.7 μM, 1.0 μM and 0.9 μM for SIRT1, respectively (Huhtiniemi et al. 2011; Mellini et al. 2013; Suzuki et al. 2009). The compound devised by Huhtiniemi et al. in Figure 16 has not been tested in cells, but the other two inhibitors are cell permeable and inhibit the proliferation of cancer cell lines (Mellini et al. 2013; Suzuki et al. 2009).

2.7.3 Small molecules

Multiple small molecule sirtuin inhibitors have been found with high throughput (HTS) or virtual screenings. Since SIRT1 has been the most extensively studied sirtuin, most inhibitor screenings have been targeted to this subtype. Nevertheless, many of the identified compounds inhibit also other sirtuins. The binding site and inhibition mechanism of most of the small molecule inhibitors remain unknown. It has been proposed that they bind to the substrate binding area in the B and C binding pockets of NAD^+ and/or the lysine binding cavity like the selisistat analogue and suramin in their respective crystal structures (Schuetz et al. 2007; Zhao et al. 2013a). However, they might also cause larger conformational changes in sirtuin structure, such as occurs with SirReal2 (Rumpf et al. 2015).

Selisistat

The only sirtuin inhibitor in phase II clinical trials, selisistat or EX-527, was discovered in a HTS campaign (Figure 17) (Napper et al. 2005). While studying the analogues in the same study, a seven-membered ring analogue displayed comparable inhibitory activity (Figure 17). Structurally selisistat belongs to indoles and its *S*-enantiomer is biologically active. It has an IC_{50}

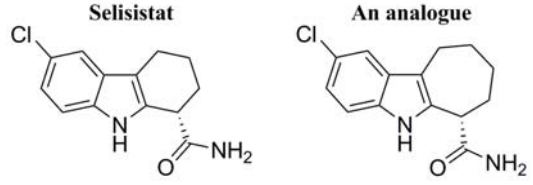


Figure 17. Selisistat (EX-527) and its analogue with a seven membered ring.

value of 100 nM for SIRT1, but it has only micromolar activity towards the other subtypes (Napper et al. 2005). Thus, selisistat is considered as a selective SIRT1 inhibitor. The crystal structure of the seven member ring containing analogue bound to the SIRT1 active site revealed partially the inhibition mechanism (Zhao et al. 2013a). The crystal structure shows that by occupying the C pocket and by making the same hydrogen bonds with the conserved residues as the nicotinamide moiety of NAD^+ (see 2.2.1 and Figure 5), the analogue sterically prevents NAD^+ from binding in the reactive conformation. Furthermore, another group demonstrated that the inhibition requires an active enzyme with both substrate and NAD^+ and subsequent release of nicotinamide (Gertz et al. 2013). Thus, selisistat works as an uncompetitive inhibitor of sirtuins, which binds to the enzyme-substrate complex during the deacetylation reaction. Since selisistat binds to conserved residues in the catalytic region of sirtuins, the structural features cannot explain the differences in the inhibitory potencies between the different subtypes. The authors have postulated that the differences arise from kinetic differences and substrate availability (Gertz et al. 2013).

In cells, selisistat inhibits SIRT1 and thus increases p53 acetylation levels, but fails to affect cell survival after DNA damage (Solomon et al. 2006). The beneficial function of selisistat as SIRT1 inhibitor in Huntington's was discovered in mammalian cells and flies, followed by mice studies, but the exact mechanism of action is not known (Smith et al. 2014). Selisistat has been demonstrated to be safe within therapeutic levels in healthy volunteers in two phase I clinical trials (Sussmuth et al. 2014; Westerberg et al. 2014).

β-naphthols

Sirtinol is the first identified sirtuin inhibitor with a β-naphthol scaffold (Figure 18). It was found in a phenotypic screening and both of its enantiomers are equally active with 48 μM IC_{50} of SIRT1 (Grozinger et al. 2001; Mai et al. 2005). Sirtinol inhibits the proliferation and induces apoptosis of lung and breast cancer cells, and it sensitizes them to other chemotherapeutic agents (Fong et al. 2014; Pal et al. 2013; Wang et al. 2012a). The sirtinol scaffold led to the synthesis of para- and meta-sirtinol and a group of analogues, which displayed improvement in inhibitory activity and

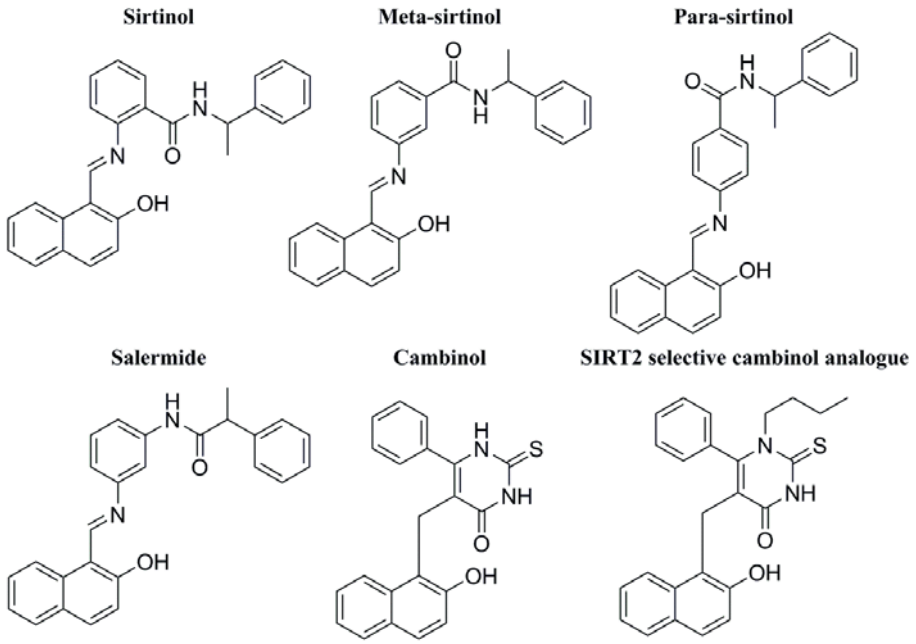


Figure 18. Examples of sirtuin inhibitors with a β-naphthol scaffold.

which also inhibit cancer cell proliferation (Mai et al. 2005; Pasco et al. 2010). Reversing the amide bond in meta-sirtinol resulted in salermide, which is more potent than sirtinol; it has been found to be able to induce apoptosis in cancer cells (Lara et al. 2009; Liu et al. 2012; Rotili et al. 2012). Initially, cambinol appears to be different from the previous inhibitors, but actually it was developed from sirtinol by multiple isosteric replacements (Figure 18) (Heltweg et al. 2006). Cambinol displays micromolar inhibition of SIRT1 and SIRT2, whereas it does not significantly affect the activity of SIRT3 and SIRT5. Recently, Mahajan et al. have published details of salermide analogues with improved sirtuin subtype selectivity and antilymphomatic activity (Mahajan et al. 2014). A SIRT2 selective analogue of this series is shown in Figure 18.

Splitomicin and derivatives

In the same year as sirtinol was described, splitomicin was found in a cell based screening (Figure 19) (Bedalov et al. 2001). Splitomicin inhibits yeast Sir2 with micromolar potency, but blocks SIRT1 only weakly (Bedalov et al. 2001). Nevertheless, the scaffold of splitomicin has been a target for extensive structure-activity relationship (SAR) studies (Neugebauer et al. 2008; Posakony et al. 2004). Posakony et al. reported details of a splitomicin analogue HR73 with a SIRT1 IC_{50} value of 5 μM (Figure 19). In 2010, some tetracyclic pyrimidinediones were reported to function as sirtuin inhibitors (Rotili et al. 2010). Structurally they resemble both splitomicin and cambinol, and they are selective towards SIRT1 with low micromolar IC_{50} values. These pyrimidinediones have been found to inhibit the proliferation of various cancer cell lines. They contain a scaffold closely resembling that of thiobarbiturates. Coincidentally, compounds with thiobarbiturate moiety were identified as sirtuin inhibitors in a virtual screening already two years earlier (Figure 19) (Uciechowska et al. 2008). In that study, the virtual screening target was the nicotinamide binding site of the yeast Sir2. The most potent thiobarbiturate compounds inhibit SIRT1 and SIRT2 with low micromolar potency. The example compound shown in Figure 19 displayed IC_{50} values of 13 μM for SIRT1 and 9 μM for SIRT2.

Urea and thiourea containing inhibitors

Suramin is a symmetric diarylurea compound, and a potent inhibitor of sirtuins; it was identified in an *in vitro* screening campaign for sirtuin activators (Figure 20) (Howitz et al. 2003). Suramin is too large itself to fit inside the binding cavity of only one sirtuin protein. The inhibitory activity and binding mechanism were explained with a SIRT5 crystal structure, where suramin was found to be symmetrically bound to the active cleft of two SIRT5 proteins (Schuetz et al. 2007). The bicyclic moiety with sulphonic acids is mainly situated in the B pocket in the NAD^+ binding site, and thus it sterically prevents NAD^+ from binding. The bound suramin stabilizes the dimer formed by the two SIRT5 proteins, which only share a small surface area with each other. Suramin

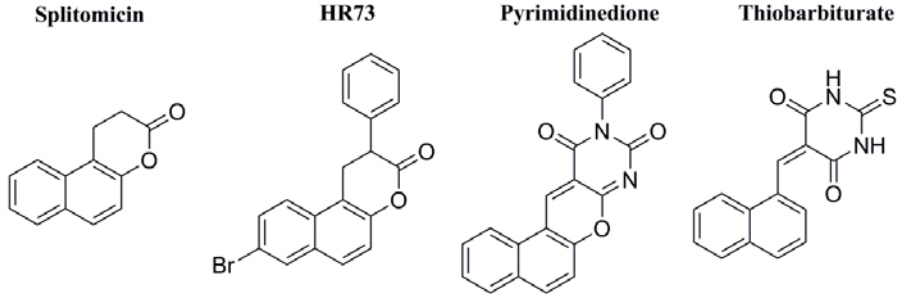


Figure 19. Splitomicin, its derivative HR73 and examples of inhibitors with pyrimidinedione and thiobarbiturate scaffold.

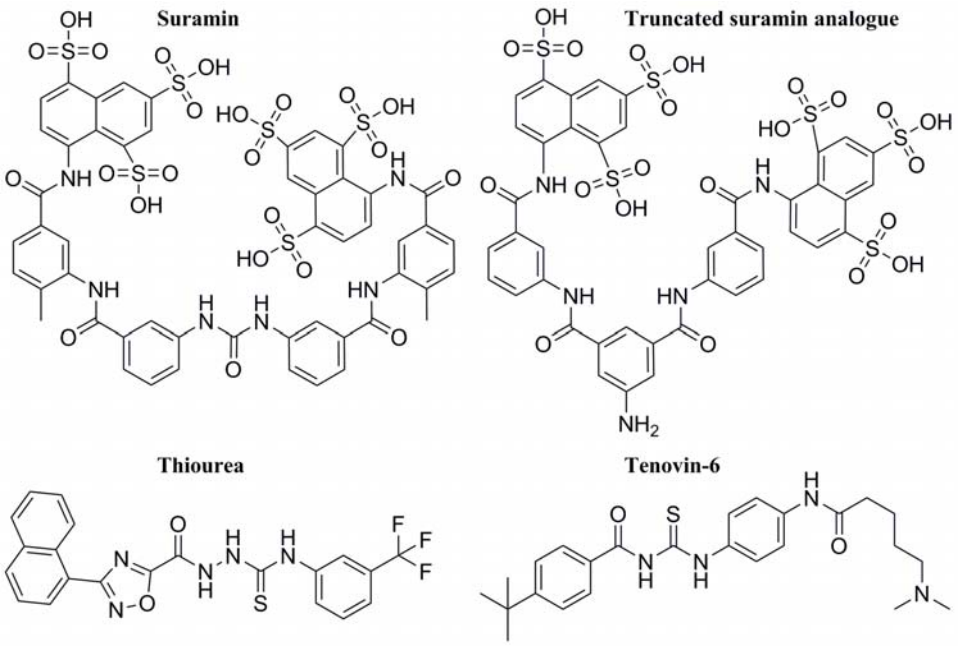


Figure 20. Suramin, its truncated analogue, an example of thiourea compounds and tenovin-6.

is used to treat parasitic infections, such as trypanosomiasis (Darsaud et al. 2004). It has problems in cell penetration because it contains many anionic groups and has a large molecular mass, and thus truncated versions of suramin have been developed (Trapp et al. 2007). Even though the truncated molecules possess a good inhibition potency (SIRT1 IC_{50} 95 nM), they still face problems in cell penetration due to their mass and anionic nature.

In addition, thiourea compounds have been identified as sirtuin inhibitors. The most potent thiourea compound resulting from a virtual screening with a SIRT1 homology model and following inhibitor design displayed an IC_{50} value of 13 μM against SIRT1 (Figure 20) (Huhtiniemi et al. 2008). Later, a cell based assay was used to discover tenovins with an acylthiourea scaffold (Lain et al. 2008; Tavares et al. 2010). Even though they were initially discovered as p53 activators, they display low micromolar inhibition with 10-20 μM IC_{50} of SIRT1 and SIRT2 (Lain et al. 2008). Tenovins are hydrophobic, which caused concerns of their *in vivo* properties, even though they inhibited the growth of multiple cancer cell lines. The more water soluble tenovin-6 has remained in the focus of research, and there are reports of its inhibitory function in various cancer cell lines (Hirai et al. 2014; MacCallum et al. 2013; Ueno et al. 2013).

Inhibitors derived from natural products

Natural products have functioned as or inspired the design of many modern inhibitor molecules, including some affecting the sirtuins (Figure 21). One research group tested two compounds; guttiferone G derived from a Vietnamese tree and hyperforin derived from St. John's wort, for their sirtuin inhibition (Gey et al. 2007). Both inhibitors displayed low micromolar IC_{50} values of 9-28 μM against SIRT1 and SIRT2. A tanikolide dimer derived from Madagascar cyanobacteria was identified as a SIRT2 selective inhibitor with an IC_{50} value of 176 nM while ensuring its stereochemistry (Gutierrez et al. 2009). A more robust approach for natural products as sirtuin inhibitors was utilized by a research group which revealed that fenugreek seed extract could act as a SIRT6 inhibitor (Yasuda et al. 2011). By using SIRT6 coated magnetic beads, they could identify two flavonoids, quercetin and vitexin as the active ingredients from this mixture of biological compounds, but the exact inhibitory activities were not determined.

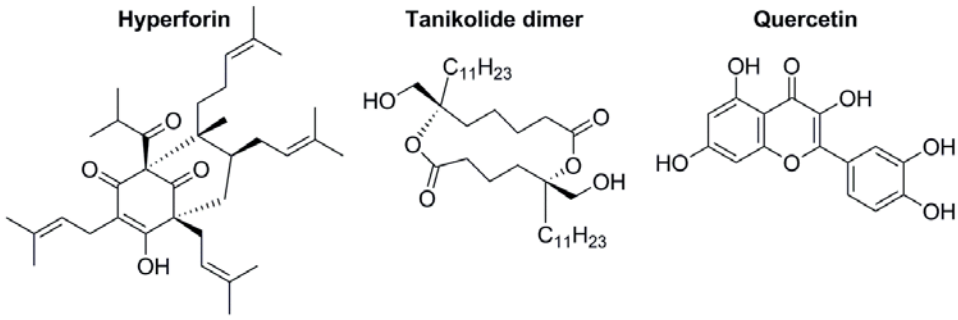


Figure 21. Examples of sirtuin inhibitors derived from natural products.

Inhibitors with various structures

In addition to the inhibitors described above, many *in silico* and *in vitro* screenings have produced structurally diverse sirtuin inhibitors, some of which will be discussed here. Virtual screening using SIRT2 substrates binding site as the target has identified inhibitors with micromolar potencies in two different studies (Figure 22) (Tervo et al. 2004; Tervo et al. 2006). Even though the found scaffolds were subjected to SAR studies, their modifications generally displayed comparable potency to the initial hits (Kiviranta et al. 2006; Kiviranta et al. 2007; Kiviranta et al. 2008). It was noteworthy, however, that the molecular size of the initial hits could be reduced without affecting the inhibitory activity.

Sometimes the inhibitors are found before their mechanism of action is known. An example is AGK2, which is a SIRT2 selective and cell permeable inhibitor with an IC_{50} value of 4 μM for SIRT2 (Figure 23) (Outeiro et al. 2007). AGK2 was initially discovered to rescue cell lines from α-synuclein toxicity, which is an important factor in Parkinson's disease. It was then decided to elucidate the mechanism of action of AGK2 with SIRT2 being included as one of the possible targets; in fact, SIRT2 inhibition was discovered to be the main mechanism of action. This finding brought selective SIRT2 inhibition to the forefront of possible new pharmacological interventions in Parkinson's. Recently, AGK2 has been shown to prevent rat astrocytes from reactive gliosis, which is one of the most important hallmark of Alzheimer's disease (Scuderi et al. 2014).

AK-7 is another another cell permeable SIRT2 selective inhibitor (Taylor et al. 2011). It was found using a substructure search of sulfobenzoic acid derivatives and this compounds has an IC_{50} value of 15 μM against SIRT2. AK-7 has displayed neuroprotective properties in mice model of Huntington's (Chopra et al. 2012). Recently a more complex SIRT2 inhibitor, AEM2, was identified in a HTS campaign for SIRT1 inhibitors (Hoffmann et al. 2014). It has the comparable inhibition potency to AGK2 and it sensitizes lung cancer cells to induced apoptosis. Other SIRT2 specific inhibitors targeting cancer are chroman-4-ones, dozens of which have been published (Friden-Saxin et al. 2012; Seifert et al. 2014). The most potent chroman-4-ones display <10 μM IC_{50}

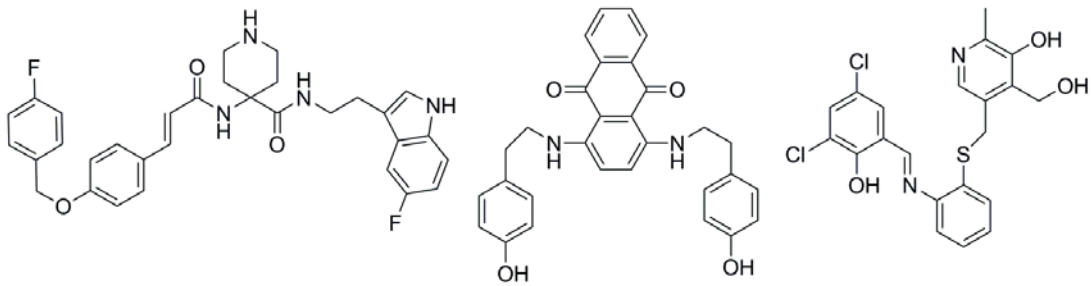


Figure 22. SIRT2 inhibitors from virtual screenings.

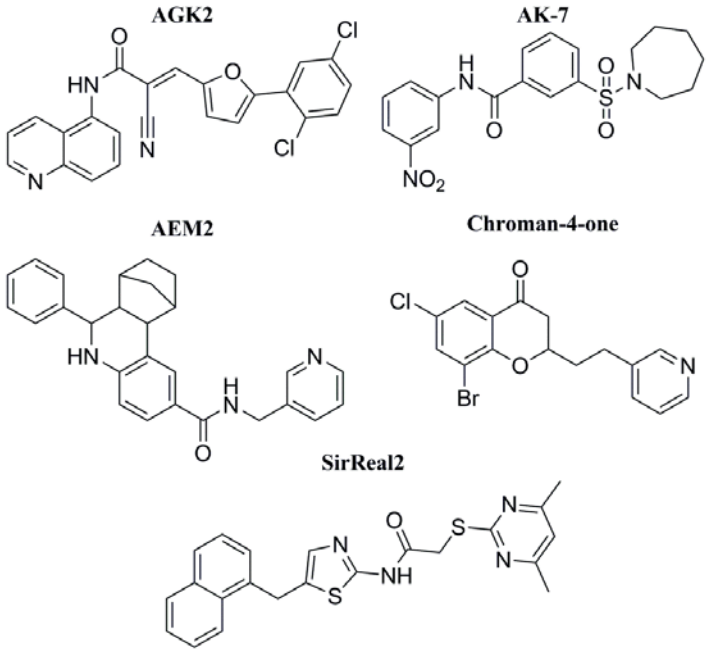


Figure 23. Examples of SIRT2 selective inhibitors.

values for SIRT2 and have been reported to inhibit the proliferation of breast and lung cancer cell lines (Seifert et al. 2014).

Very recently, SirReal2 was identified as a SIRT2 selective inhibitor in an *in vitro* screening (Figure 23) (Rumpf et al. 2015). It displays an IC_{50} of 140 nM for SIRT2 with marginal effects on other sirtuins; for example, SIRT6 activity is inhibited by SirReal2 only 19% at 200 μM. The crystal structures of SirReal2 and NAD^+ bound together to SIRT2 reveal a unique binding site for this inhibitor. NAD^+ is bound in the productive conformation with the nicotinamide moiety in the C-pocket, while the inhibitor is bound to the zinc binding domain in the substrate binding region. This forces the zinc binding domain orientation to resemble the inactive sirtuin conformation despite the presence of the bound cofactor. The inhibitor also sterically disturbs the structure of the Ac-lysine binding tunnel and hinders hydrogen bond formation between the lysine N^{ε} and the Val residue (see 2.2.1 and Figure 5). It remains to be determined if this binding mode is specific for SIRT2 or whether it is common for all (or some) sirtuin subtypes. SirReal2 also caused α-tubulin hyperacetylation in cells, which is a commonly observed phenomenon when SIRT2 is inhibited *in vivo*.

3 Aims of the Study

The general aim of this doctoral thesis was to utilize *in silico* and *in vitro* methods in studying sirtuin inhibitors. This included an investigation of the action of known sirtuin inhibitors as well as the discovery and design of novel sirtuin inhibitors.

The more specific aims were as follows:

1. To gain insights into SIRT6 inhibition by peptides, pseudopeptides and small molecules. (I, II)

2. To investigate the *in vitro* deacetylation activity of SIRT6 with different fluorophoric substrates. (II)

3. To build a three dimensional quantitative structure-activity (3D QSAR) model of the peptidic and pseudopeptidic SIRT1 inhibitors and to use this model in the design of new inhibitors. (III)

4. To investigate if a new putative binding site of SIRT3 can be used in virtual screening of sirtuin inhibitors. (IV)

4 Methods

Only a brief overview of the methodology is given here. For the more detailed methodology the reader is referred to the methods sections in publications I-IV or their corresponding supplementary materials.

4.1 COMPUTATIONAL METHODS

The computational methods of this thesis included homology model construction, molecular docking and 3D QSAR modelling.

4.1.1 Structure preparation (I, III, IV)

The protein models to be used in docking in studies I and III and in virtual screening in study IV were pre-processed with the Protein Preparation Wizard of Schrödinger Suite. Bond orders were assigned and possibly missing hydrogens and/or side chains were added, and possible waters >5 Å away from the heteroatoms were removed. The Optimized Potentials for Liquid Simulations OPLS2005 force field was used to calculate the partial charges (Banks et al. 2005). Impref minimization (conjugate-gradient) was performed with heavy atom converged to RMSD 0.30 Å.

The inhibitors to be docked were either drawn and converted to 3D with Discovery Studio, Molecular Object Environment (MOE) or Maestro (I, IV), or converted to 3D with X-Concord from ChemBioFinder derived sdf-files (III). The ZINC database for the virtual screening in study IV was downloaded from the Internet. In all cases, the 3D structures of the molecules were pre-processed with LigPrep to generate possible tautomers in pH 7 ± 2, to desalt them and to calculate the partial charges with force field OPLS2005 (Banks et al. 2005).

4.1.2 Homology modelling (I)

Since the SIRT6 crystal structure displays the open and inactive conformation, which is unsuitable for docking of the peptidic and pseudopeptidic inhibitors, a homology model was created to study the interactions of the inhibitors with SIRT6 in study I (Pan et al. 2011). The homology model was constructed using the ORCHESTRAR tool in Sybyl-X. The SIRT3 crystal structure with bound N^{ε}-thio-Ac-lysine inhibitor (PDB ID: 3GLR) was used as a template, because it is in a biologically active conformation, which enables the docking of substrate based inhibitors (Jin et al. 2009). The SIRT6 catalytic domain amino acid sequence was aligned with the SIRT3 PDB sequence, and it displayed 40% identity. The backbone of the homology model was constructed according to the template whenever possible, and missing loops were predicted using the CODA library (Deane and Blundell 2001). The side chains of the homology model were added either from the template or from a rotamer library, while avoiding steric clashes. All hydrogens were added and partial charges were calculated using the Assisted Model Building with Energy Refinement AMBER7_FF9 force field (Duan et al. 2003). Finally, the obtained homology model of SIRT6 was minimized with staged minimization using the conjugate-gradient Powell method (Powell 1964).

4.1.3 Molecular docking (I, III, IV)

Molecular docking was used to study the interactions between inhibitors and sirtuins. With the SIRT6 homology model, the GRID-file for the Glide docking was calculated to fit ligands ≤ 20 Å in length from the centre of residues Trp187 and Pro219, which describe the Ac-lysine binding cavity. Constraint was made to ensure the H-bond formation between Ac-lysine analogue and

the backbone carbonyl oxygen of Leu184 (corresponding to Val292 in Figure 5). Similarly, the GRID-file of the Ac-lysine binding site of SIRT1 crystal structure was constructed from the centre of residues His363, Phe414, Asn417 and Arg446 (Zhao et al. 2013a). Also here, the H-bond constraint with Val412 was used to guide the inhibitors to adopt their assumed binding pose. Molecular docking in studies I and III was performed with standard precision (SP) Glide (Friesner et al. 2004). The dockings were performed with the corresponding constraints, and after the docking the best scoring poses were subjected to post-docking minimization.

For the virtual screening of SIRT3 inhibitors in study IV, the GRID-file was calculated from the centre of residues Phe186, Gln260, Asp290 and Glu296 with no constraints. Glide high throughput virtual screening (HTVS) was used in the initial screening of the pre-processed ZINC database, and the top 5% scoring poses were refined with the Glide SP run. From the SP run the top 5000 poses were further refined with an extra precision (XP) run. The XP docking results were visually inspected to select compounds for *in vitro* testing.

4.1.4 Quantitative structure-activity relationships (III)

The relationship between the *in vitro* activity and the 3D molecular interaction fields of the inhibitors was studied using comparative molecular field analysis (CoMFA) (Cramer et al. 1988). The 3D structures of the inhibitors were used in their biologically active conformation, which were obtained either by docking or alignment with a reference compound. For the CoMFA model the charged amino and carboxylic acid groups were neutralized and semi-empirical AM1 partial charges were added using single point calculation with MOPAC (Dewar et al. 1985; Tsai et al. 2010). Multiple descriptor type, variable and partial least squares (PLS) component amount optimizations finally resulted in the model presented in study III. The predictivity of the model was explored with the methods of Chirico and Gramatica (Chirico and Gramatica 2011; Chirico and Gramatica 2012). These methods were used to scrutinize the differences between the observed and predicted activities while taking also the regression curve angle into consideration.

4.2 *IN VITRO* METHODS

The *in vitro* activities of the inhibitors in studies I-IV were determined with fluorescence based assays (Figure 24). The sirtuin activity assays are based on an assay substrate, which includes a peptide with N^{ε}-Ac-lysine. A fluorophore, 7-amino-4-methylcoumarin (AMC), is linked to the carboxy terminus of the N^{ε}-Ac-lysine. During the incubation, the sirtuin deacetylates the substrate in the presence of cofactor NAD^{+}. After the incubation sirtuin activity is inhibited with an excess of nicotinamide and trypsin as a developer solution is added to cleave off AMC from the deacetylated substrate. This produces fluorescence, the amount of which is directly proportional to sirtuin deacetylation activity.

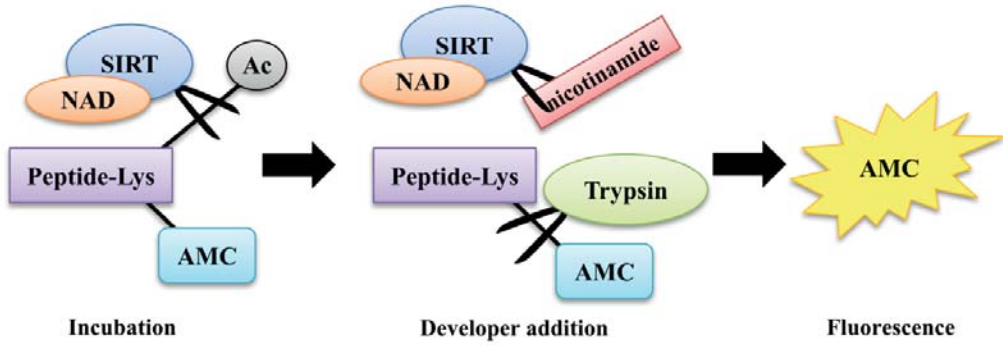


Figure 24. The principle of the fluorescence based deacetylation assays.

4.2.1 SIRT1-3 (I, III, IV)

The *in vitro* assays of SIRT1-3 were based on the product sheets of Enzo Life Sciences. The SIRT1 assay substrate is based on residues 379-382 of p53 (KI177), and the SIRT2/3 assay substrate is based on residues 317-320 of p53 (KI179). The assay buffer (KI286) contained 50 mM tris(hydroxymethyl)-aminomethane-hydrochloride (Tris-HCl), 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2 and 1 mg/ml bovine serum albumin. The assays included the assay substrates at 0.7 K_m and NAD^+ at 0.9 K_m. The assay components excluding the recombinant enzyme were pipetted to half-volume 96 plate wells with 2.5 µl of inhibitor in DMSO or DMSO (the latter being the uninhibited control reaction). The reactions were started by the addition of the recombinant enzyme and the reaction mixtures (total 50 µl) were incubated at 37 °C for 60 minutes. The reactions were terminated by the addition of 50 µl of 2 mM nicotinamide (KI283) and developer solution (KI176). The terminated reaction mixture was incubated further at 37 °C for 45 minutes. Finally, the fluorescence readings were obtained from an EnVision 2104 Multilabel Reader using 370 nm and 460 nm for excitation and emission, respectively.

4.2.2 SIRT6 (I, II)

The SIRT6 *in vitro* deacetylation assay used in study I was based on Cayman product sheet (item 700290). The assay substrate is based on residues 379-382 of p53 (700293). The assay buffer contains 50 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl and 1 mM $MgCl_2$ (700291). The buffer (700291), the SIRT6 recombinant enzyme (700292), 5 µl of the inhibitor in DMSO or DMSO (the latter being the uninhibited control reaction) were pipetted into the black half-volume 96 well plates. The SIRT6 assay was started by adding the assay substrate and NAD^+ solution (400 µM and 3 mM in the reaction solution, respectively). The reaction mixture was incubated at 37 °C for 90 minutes, and the reaction was terminated with nicotinamide (700295) and developer solution (700296). The terminated reaction mixture was incubated at room temperature for 30 minutes. The fluorescence readings were obtained with Victor 1420 Multilabel Counter (Wallac, Finland) with 370 nm and 430 nm for excitation and emission, respectively.

In study II, the fluorophoric substrate Ac-Arg-Tyr-Gln-Lys(Ac)-AMC was used in inhibitor screening. The reaction mixture included 320 µM of the above substrate, 3 mM of NAD^+ at the SIRT6 assay buffer (Cayman 700293), 4.5 µg/well of recombinant SIRT6 enzyme and 2.5 µl of inhibitor in DMSO or DMSO (total volume 50 µl). The reaction was initiated with the addition of enzyme and incubated at 37 °C for 90 minutes, and terminated by addition of nicotinamide and trypsin solution. The terminated reaction was incubated at room temperature for 30 minutes. The fluorescence readings were obtained with EnVision 2104 Multilabel Reader using 380 nm and 440 nm for excitation and emission, respectively.

4.2.3 Signal interference determination (IV)

The signal interference of a group of inhibitors examined in study IV was determined to ensure that the compounds actually inhibited SIRT3. It was possible that they could cause signal interference due to their physico-chemical properties, such as autofluorescence or colour, which could absorb the wavelengths used in the assay. In order to exclude this possible artefact, the commercial assay kit from Enzo Life Sciences provides a deacetylated standard (KI142). The deacetylated standard produces fluorescence with the addition of developer/trypsin solution, and it should correspond to the substrate deacetylated by sirtuin. To test the signal interference of the inhibitors at their tested concentration (200 µM), standard curves were determined with four different concentrations of deacetylated standard (0 µM, 1.25 µM, 2.5 µM and 5 µM), which covered the observed fluorescence range of used in the sirtuin assays. The solvent or inhibitor in solvent together with the deacetylated standard was pipetted into wells with appropriate dilutions to maintain the DMSO concentration used in the assay. The fluorescence readings were obtained at the same wavelengths and equipment as used in the SIRT1-3 assay.

5 Main Results and Discussion

The main results of this thesis include insights into SIRT6 deacetylation together with the design and screening of new substrate based and small molecule sirtuin inhibitors.

5.1 SIRT6 DEACETYLATION INHIBITION (I, II)

Among its diverse enzymatic activities, SIRT6 undertakes deacetylation of H3K9, H3K56 and DNA endonuclease RBBP8 (Kawahara et al. 2009; Michishita et al. 2008; Yang et al. 2009). In studies I and II, the deacetylation reaction catalysed by SIRT6 was studied with known sirtuin inhibitors *in vitro*. This was undertaken as the starting point for investingating SIRT6 inhibitors, since there were only a few SIRT6 inhibition studies and no SAR discussions available before studies I and II. In study I, the first structure-activity relationship study of the inhibition of SIRT6 deacetylation was conducted using 16 substrate based inhibitors of SIRT1 and SIRT2. The inhibitors consisted of thio-Ac-lysine peptides (inhibitors **1**-**7** in Table 2), branched N^{ε}-modification containing tripeptides (**8**-**10**) and potent pseudopeptidic SIRT1 and SIRT2 inhibitors (**11**-**16**). All of the tested compounds inhibited the deacetylation reaction of SIRT6. The inhibition activities of the compounds were generally relatively weak at 200 μM concentration, but three compounds displayed >60% inhibition (Table 2).

The thio-Ac-pentapeptides based on p53 sequence (**2**) and its alanine replacement (**3**) displayed superior inhibitory activity to the thio-Ac-tripeptides (**4**-**7**) and the thio-Ac-pentapeptide based on the α-tubulin sequence (**1**). The most potent thio-Ac-tripeptide **5** displayed mediocre activity with alanine residues in its C- and N-terminus, whereas the more bulky tripeptides **4**, **6** and **7** exhibited slightly lower inhibitory activities. The thio-Ac-pentapeptides **1**-**3** possessed better inhibitory activities, possibly because they have a larger contact surface and more interaction possibilities with the enzyme than the shorter tripeptides. The α-tubulin based inhibitor **1** did not have much activity towards SIRT6 possibly because α-tubulin is a target of SIRT2 deacetylation.

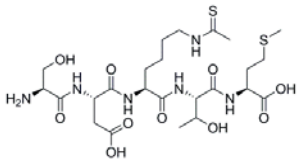
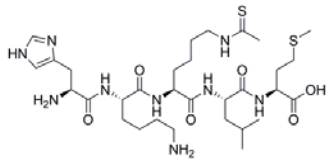
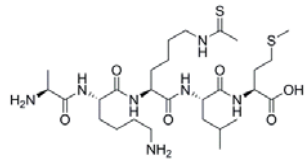
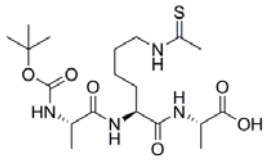
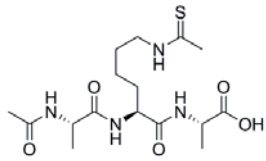
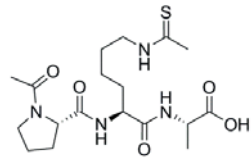
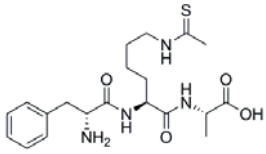
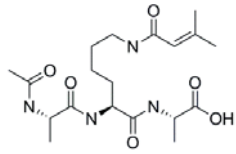
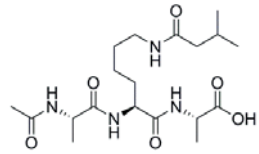
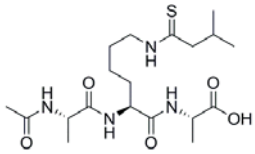
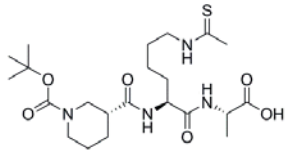
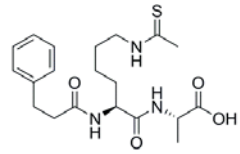
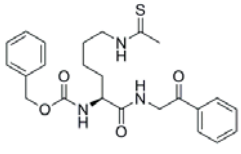
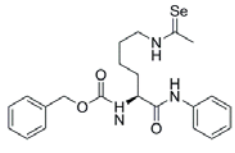
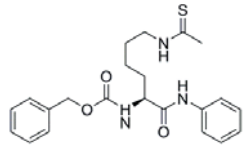
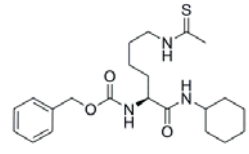
The branched N^{ε}-modification containing tripeptides **8**-**10** displayed poor inhibition of SIRT6. This is somewhat conflicting with the observation that SIRT6 is also able to deacylate N^{ε}-modifications longer than Ac, such as myristoyl, and successful inhibitors have been developed from them (see 2.7.2) (He et al. 2014). It seems that the lysine binding site of SIRT6 cannot accommodate branching acyl modifications as well as longer but non-branching modifications.

The most potent pseudopeptides **11**, **12** and **14** of study I displayed similar inhibitory activity to the most potent thio-Ac-tripeptide **5**. They contain modifications not present in natural peptides, and thus this result indicates that it is possible to develop synthetic SIRT6 inhibitors with comparable activity to the shorter natural peptides. The properties of the non-natural modifications affected the inhibitory activity; compound **13**, which has an aromatic group with a longer carbonyl linker on its C-terminal side chain, was slightly more potent than its counterpart **15**, which has a similar aromatic group with a shorter linker. It is noteworthy, however, that these minor differences of activity might not be statistically significant and possible to see in IC_{50} values.

Molecular docking was used to study if the differences in the inhibitory activities could be explained by the differences in interactions of the compounds with SIRT6. Even though the crystal structure of SIRT6 was available when study I was being undertaken, it could not be used in the molecular docking due to its inactive open conformation (Pan et al. 2011). Instead, a homology model was constructed using a SIRT3 structure as the template (Jin et al. 2009). The high identity (>30%) of the SIRT3 and SIRT6 catalytic domain sequences indicated that homology modelling would possibly be successful (Cavasotto and Phatak 2009). Furthermore, the major

differences observed in the amino acid sequences of SIRT3 and SIRT6 were not close to the lysine binding region of interest. Indeed, the constructed homology model of the substrate binding region was in its biologically active conformation and 14 out of the 16 inhibitors could be docked into this model. For compound **1** no poses were returned from the docking run and compound **14** was excluded from docking because molecular modelling softwares lack capabilities for handling selenium.

Table 2. The inhibition activities of peptidic and pseudopeptidic compounds against SIRT6 deacetylation at 200 μM. The mean and standard deviation (SD) of the inhibition percentages were calculated from at least three determinations.

No.	**1**	**2**	**3**
Structure			
%±SD	35 ± 0.3	62 ± 9	91 ± 6
No.	**4**	**5**	**6**
Structure			
%±SD	40 ± 6	54 ± 10	32 ± 16
No.	**7**	**8**	**9**
Structure			
%±SD	33 ± 16	24 ± 2	23 ± 2
No.	**10**	**11**	**12**
Structure			
%±SD	45 ± 14	59 ± 5	51 ± 14
No.	**13**	**14**	**15**	**16**
Structure				
%±SD	44 ± 4	62 ± 5	36 ± 6	30 ± 10

All the successfully docked inhibitors displayed the β staple binding conformation (described in 2.2.2) with hydrogen bonds both to the zinc binding and the Rossmann fold domains. When the docking poses were examined, it was observed that the pentapeptides indeed had more hydrogen bonding opportunities with the side chains of SIRT6 than the shorter peptides. The most potent pentapeptide **3** formed two extra hydrogen bonds: between its lysine and the carbonyl oxygen of Glu187 and between its valine carbonyl and the amide nitrogen of Arg218. The most potent thio-Ac-tripeptide **5** also had an extra hydrogen bond with its carboxyl group and the amide nitrogen of Arg218 (Figure 25). This hydrogen bond is also present in the branched N^{ϵ}-modification containing tripeptides **7**-**9**, even though their inhibitory activity is low. Their bulky N^{ϵ}-modifications might collide with Trp69, which is situated at the active cleft between N^{ϵ}-Ac and NAD^{+} binding sites. The corresponding residue in SIRT1-3 is Phe, which is smaller and can better accommodate branching substituents. Differences of inhibitory activity of the pseudopeptidic compounds could also be explained by the docking to some extent. The longer carbonyl chain linked aromatic ring of inhibitors **13** can participate in π-π interactions with Trp186, whereas inhibitor **15** with shorter linked aromatic moiety cannot reach the residue.

SIRT6 deacetylation activity was observed to be weak when compared to that of the other sirtuins during the *in vitro* tests of study I. This has been observed before by other groups (Hu et al. 2013; Pan et al. 2011). However, since the deacetylation by SIRT6 has significant functions *in vivo* (see 2.4.2); the deacetylation activity *in vitro* was examined in more detail. In study I, the observed fluorescence of the deacetylation activity assay was slightly increased with prolonged incubation time and optimized excitation and emission wavelengths. Since the deacetylation catalysed by SIRT6 is highly substrate specific, the subsequent experiments were focused on the fluorogenic assay substrates. The p53 based fluorogenic substrate might not be ideal for studying SIRT6 deacetylation, because p53 is not a natural substrate of SIRT6. In study II, four different AMC-labelled substrates were designed to check the impact of the assay substrate on the deacetylation reaction. These compounds were based on the potent inhibitors identified in study I and sequences of SIRT6 natural substrates H3K9 and H3K56 (Kawahara et al. 2009; Michishita

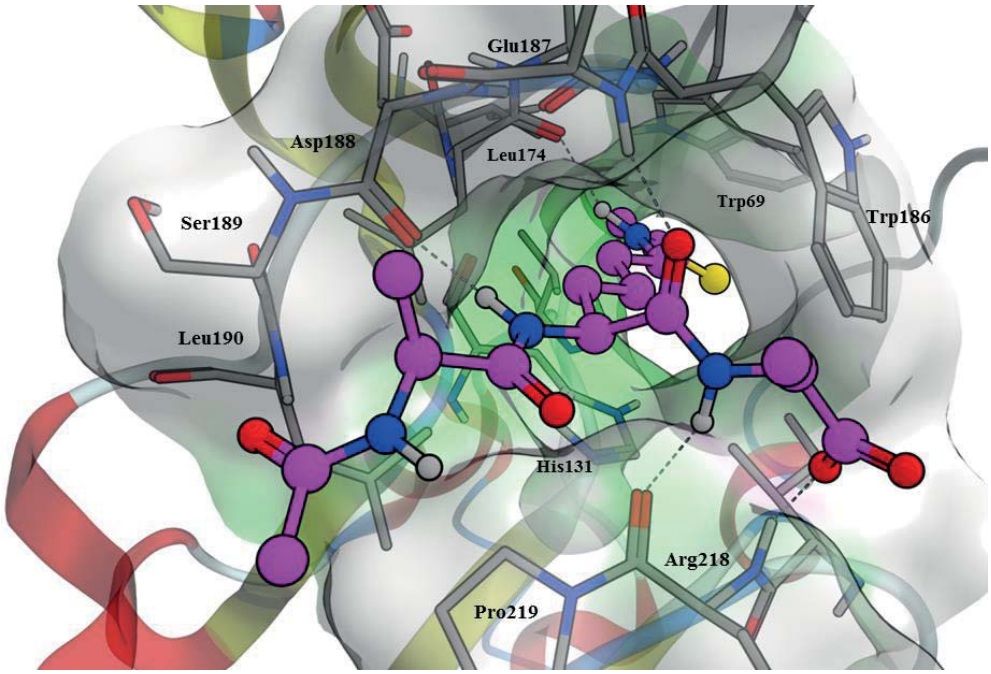
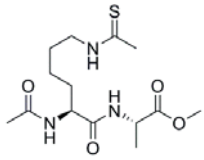
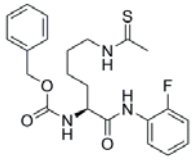
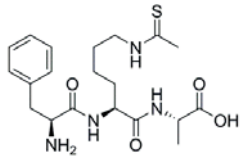
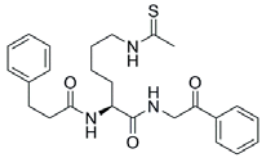
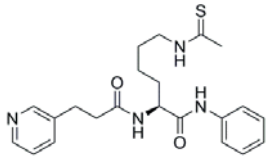
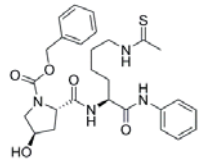
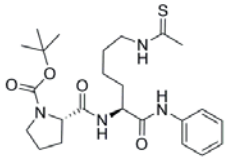
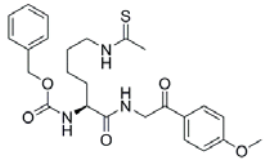
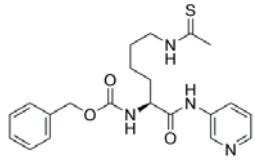
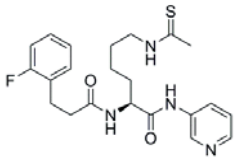
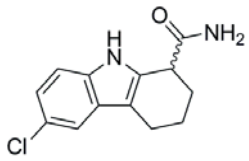
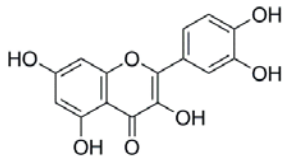


Figure 25. The interactions of inhibitor **5** with the SIRT6 homology model. The structure was rendered with Molecular Operating Environment MOE v. 2014.3.

et al. 2008; Yang et al. 2009). The substrates used in study II were Ac-Arg-His-Lys-Lys(Ac)-AMC (based on p53 residues 379-382), Ac-His-Lys-Lys(Ac)-AMC (based on p53 residues 380-382), Ac-Ala-Lys-Lys(Ac)-AMC (based on inhibitor **3**), Ac-Thr-Ala-Arg-Lys(Ac)-AMC (based on histone 3 residues 6-9) and Ac-Arg-Tyr-Gln-Lys(Ac) (based on histone 3 residues 53-56). The emission and excitation wavelengths were optimized individually for each substrate in the initial substrate screening. The substrates were incubated with SIRT6 and NAD^+ in assay conditions to determine which of them would produce the most intense fluorescence. Interestingly, the natural substrate H3K56 based assay substrate emitted the highest fluorescence, indicating it was being catalysed more rapidly than the other tested substrates.

The assay parameters, such as incubation time and enzyme amount were then determined for this substrate and it was used in further screening of SIRT6 deacetylation inhibitors. Furthermore, the effects of non-specific binding partners, such as bovine serum albumin, and the addition of free fatty acids were examined. The non-specific binding partners are routinely used in *in vitro*

Table 3. The inhibition activities of pseudopeptidic inhibitors, selisistat and quercetin against SIRT6 determined with the H3K56 based assay substrate at 200 μM. The mean and SD of the inhibition percentages were calculated from at least three determinations.

No.	17	18	19
Structure			
%±SD	20 ± 4	25 ± 4	20 ± 2
No.	**20**	**21**	**22**
Structure			
%±SD	20 ± 8	18 ± 2	56 ± 8
No.	**23**	**24**	**25**
Structure			
%±SD	32 ± 2	48 ± 5	54 ± 1
No.	**26**	**Selisistat**	**Quercetin**
Structure			
%±SD	58 ± 2	56 ± 8	52 ± 4

assays to increase the stability of enzymes and the free fatty acids have increased SIRT6 deacetylation activity *in vitro* in another assay (Feldman et al. 2013). Unfortunately, none of the tested methods could increase the deacetylation activity of SIRT6 in the current assay conditions. The assay was used in screening of inhibitors without the addition of non-specific binding partners.

An IC_{50} value of 2.2 mM was determined for nicotinamide against SIRT6 using the assay based on the H3K56 substrate. This value is larger than those reported for SIRT1 and SIRT2, and it implies that SIRT6 deacetylation might not be inhibited by physiological concentrations of nicotinamide (~100 μM) (Yang and Sauve 2006). In order to study study the inhibition of SIRT6 H3K56 deacetylation further, 10 pseudopeptides and four known sirtuin inhibitors; suramin, selisistat, sirtinol and quercetin were tested in the H3K56 assay (Table 3). All of the pseudopeptides, selisistat and quercetin inhibited SIRT6, but suramin and sirtinol did not display evidence of inhibition at 200 μM.

It is noteworthy, that the inhibition percentages of study I and II are not directly comparable, since the assay conditions are different. Three of the pseudopeptides (**22**, **25** and **26**) displayed >50% SIRT6 deacetylation inhibition, which supports the possibility of developing potent pseudopeptidic SIRT6 inhibitors. The three potent pseudopeptidic inhibitors contain a short linked aromatic ring in their C-terminal, which is too short to reach the π-π interactions with Trp186. Both the C- and N-terminal modifications affect the inhibitory activity of the pseudopeptidic inhibitors. It would be interesting to check the possible combinations of the potent C- and N-terminal modifications to discover potent pseudopeptidic inhibitors of SIRT6.

In summary, the assay substrate based on H3K56 utilizing *in vitro* assay can be used for the screening of SIRT6 deacetylation inhibitors. Many known sirtuin inhibitors also have an effect on the SIRT6 deacetylation, even though they seem to be slightly more potent against SIRT1-3. However, not much is known about the effect of the inhibitors on other SIRT6 enzymatic activities. It would be most interesting to elucidate the effect of the identified inhibitors on the deacylation or mono-ADP-ribosylation activities of SIRT6.

5.2 NEW SUBSTRATE BASED INHIBITORS (III)

During the last decade tens of N^{ε}-Ac-lysine analogue containing inhibitors have been published by our group and others (Fatkins et al. 2006; Huhtiniemi et al. 2011; Kiviranta et al. 2009; Mellini et al. 2013; Suzuki et al. 2009). The lack of structural data and comprehensive SAR studies has long been a problem impeding the rational design of substrate based sirtuin inhibitors. Study III was the first to exploit the modern method of 3D QSAR in the design of new pseudopeptidic sirtuin inhibitors.

3D QSAR can be used to study the correlation of molecular properties and observed biological activity. Readers interested in the theoretical background and various aspects of 3D QSAR are referred to recent reviews (Cherkasov et al. 2014; Damale et al. 2014; Verma et al. 2010). In this study, the alignment dependent descriptor method CoMFA was used (Cramer et al. 1988). In CoMFA, the compounds are placed in a generally 2 Å spaced 3D grid lattice to interact with a chemical probe (sp^3-hybridized carbon with +1 charge) and the electrostatic (Coulombic) and steric (van der Waals) interaction energies in every grid point are calculated. Then, a PLS algorithm is used to correlate the interaction data with the biological activity to form a multidimensional QSAR equation. Subsequently, similar interaction data can be calculated for new compounds in the same lattice, and then their biological activity can be predicted with the obtained QSAR equation.

Since most of the *in vitro* data has been obtained for SIRT1, this subtype was chosen as the target of the QSAR study. Some inhibitors needed to be excluded from the study due to their uncertain docking results (pentapeptides) or possibly missing parameters in molecular modelling

softwares (e.g. selenium containing inhibitors). Pentapeptides were excluded because they can display multiple plausible β staple docking poses where the side chain interactions differ, which makes it is difficult to objectively choose only one pose for the CoMFA study. The inhibitors investigated in study III included only compounds for which the activity had been determined in our laboratory using the same procedure, which makes the comparison of inhibition activities more reliable. Due to the costs and since it was not scientifically essential, the IC_{50} values were not measured for all the compounds, especially those with low inhibition percentages in the initial tests. In order to ensure that the model creation would not be confined only to the inhibitors with measured IC_{50} values, an approximated IC_{50app} value was calculated for the inhibitors when needed. Equation 1 shows how the IC_{50app} value was calculated; the equation is derived from the Langmuir isotherm equation (Copeland 2000):

$$IC_{50app} = \frac{concentration * inhibition\ percentage}{1 - inhibition\ percentage} \quad \text{Equation 1.}$$

Ultimately, 79 inhibitors were chosen for the QSAR study and they were divided into training and test sets of 65 and 14 compounds, respectively.

The CoMFA method is extremely sensitive to the alignment of the compounds. The compounds need to be aligned in their biologically active conformation if one wishes to obtain more useful models. The biologically active conformation of substrate based sirtuin inhibitors is the β staple binding pose described in 2.2.2. Conveniently, the crystal structure of SIRT1 in biologically active conformation was published and it could be directly used in the docking of substrate based inhibitors (Zhao et al. 2013a). The best scoring poses of the compounds displaying the β staple binding were collected for further analysis. The compounds failing to dock were aligned with a reference compound, which displays β staple binding conformation, to place them into a comparable conformation.

The atomic point charges for the aligned inhibitors were calculated with the semi-empirical AM1 method, which has been shown to produce the most predictive CoMFA models (Tsai et al. 2010; Zhang et al. 2011). TheCoMFA model was constructed with the steric and electrostatic CoMFA interaction fields of the training set inhibitors. The optimum number of PLS components was determined with cross-validation and Leave-One-Out studies. The final model was built with three PLS components. In order to ensure the stability of the model with perturbations of the activity value the progressive scrambling test (perturbations of the activity values) was performed (Clark and Fox 2004). The average cross-validated coefficient of determination Q^2 of ten progressive scrambling runs was 0.32, which is only slightly lower than the 0.35 mentioned in the literature for robust models (Clark and Fox 2004). The traditional coefficient of determination R^2 (0.86), standard error of estimate (SEE) (0.48 log units) and external coefficient of determination R^2_{ext} (0.84) displayed that the model could be considered as being statistically valid. To further assess the external predictivity of the CoMFA model the validation methods devised by Chirico and Gramatica were utilized (Chirico and Gramatica 2011; Chirico and Gramatica 2012). The observed and predicted inhibitory activities were in good agreement according to the validation methods. Furthermore, the regression line angle displayed no significant deviation from 45°.

The final CoMFA equation was 58% explained by steric and 42% by electrostatic descriptors. The CoMFA contours were visually compared with the substrate binding site of SIRT1 (Figure 26). The contours are consistent with the shape and properties of the substrate binding site. A large steric bulk disallowing region (yellow in Figure 26) can be observed beyond the Ac binding site. It demonstrates the detrimental effect of larger N^{ε}-modifications on the inhibitory activity. The large steric bulk allowing region (green in Figure 26) visible near to the carbonyl oxygen of the Ac arises from the superior activity of N^{ε}-thio-Ac inhibitors to their N^{ε}-Ac counterparts. The CoMFA model cannot distinguish between different mechanisms of inhibition and thus it probably assumes that the slightly larger size (~18% increase in van der Waals' volume) of the

sulphur atom is responsible for the difference in the inhibitory activity. It is highly doubtful that the inhibitory activity would increase by replacing the carbonyl oxygen of the Ac with some bulky modification, since there is not sufficient space in the enzyme structure at that region. This finding demonstrates the importance of finding biological meaning for the CoMFA contours. In the N-terminal side of the inhibitors, a negative charge allowing contour (red in Figure 26) is present near His423. Thus, hydrogen bonding with this residue seems beneficial for the inhibitory activity. There are also two steric bulk allowing regions on the N-terminal side of the inhibitors (green in Figure 26), which implies that branching inhibitors should have better inhibition activity. Surprisingly, no clear contours are present on the C-terminal side of the inhibitors. This could arise from the smaller amount of variable C-terminal structures examined here.

To test the predictive limits of the CoMFA model, the CoMFA contours were used in designing 13 new thio-Ac containing pseudopeptidic SIRT1 inhibitors. The N-terminals of the inhibitors were designed to occupy either one or both of the steric bulk allowing regions and some of them possessed the possibility to form hydrogen bonds with His423. In addition, a variety of C-terminal modifications were used to study if the model contained information about their effect on the inhibitory activity even though this was not visible as clear contours. The designed inhibitors had a predicted pIC_{50} values in the range of 4.9-6.0 (Table 4). The inhibitory activities of the new inhibitors were determined *in vitro*. Inhibitors with *N*-boc protected proline or hydroxyl-proline in their N-terminal (**30**, **32** and **34**) could occupy both sterically allowed regions

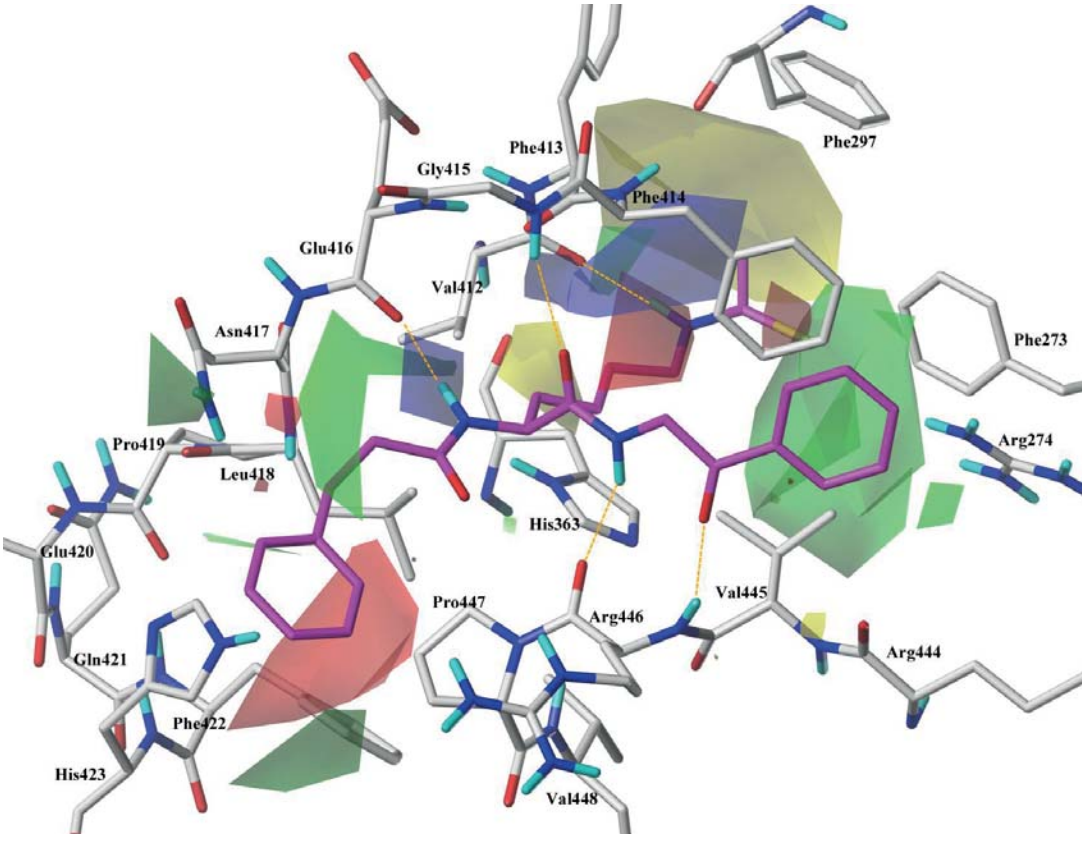
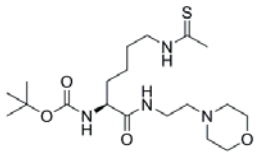
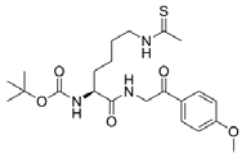
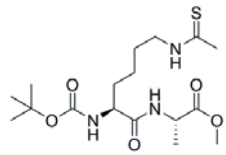
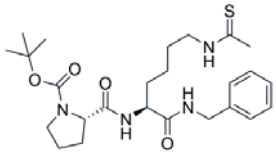
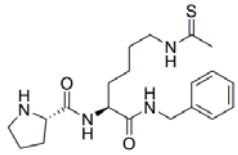
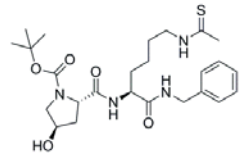
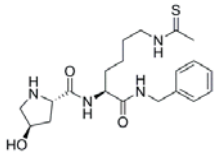
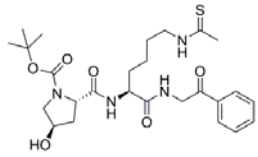
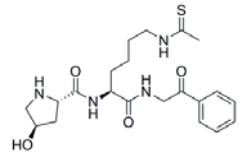
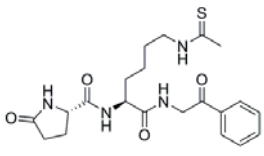
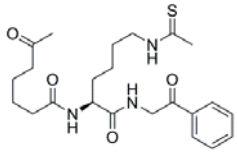
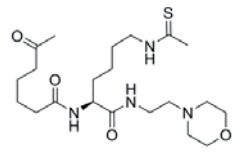
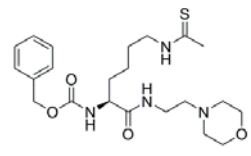


Figure 26. The interactions of an inhibitor with SIRT1 overlaid with the CoMFA contours. Colour coding for the contours: green and yellow = steric bulk allowed and disallowed regions, red and blue = negative and positive charge allowed regions, respectively. The image was rendered with Sybyl-X version 2.1. The figure was originally published in ACS Medicinal Chemistry Letters 12: 969-974, 2012 (study III). Reused with permission of the American Chemical Society.

on the N-terminal side of the inhibitors. The hydroxyl-proline inhibitors **32** and **34** also bring an electron-rich carbon to the negative charge allowing region near to His423. The *N*-boc protected inhibitors (**30**, **32** and **34**) had generally higher predicted inhibitory activities than their deprotected counterparts (**31**, **33** and **35**). It was observed that the deprotected inhibitors were docked in a different conformation than their protected counterparts, which significantly affected their predictions even though the structures represent each other in 2D. The proline ring of the deprotected inhibitors is almost perpendicular when compared to their protected counterparts. Thus, they do not occupy either of the two steric bulk allowing regions. Interestingly inhibitors **37** and **38** with a carbonyl at the negative charge allowing region near to His423, did not exhibit potent inhibitory activity either in the predictions or *in vitro*. It seems that if a compound occupies both steric bulk allowing regions at the N-terminal, this will affects its inhibitory activity more than introducing the carbonyl at the negative charge allowing region.

Table 4. The predicted (P) and experimentally derived (E) inhibitory activities (pIC_{50}) of the inhibitors designed with the CoMFA model. The means of experimentally derived inhibition percentages at 50 μM were calculated from at least three determinations and converted to pIC_{50} with Equation 1.

No.	**27**	**28**	**29**
Structure			
pIC_{50}	P = 5.0, E =4.1	P = 5.4, E = 5.6	P = 4.8, E = 4.9
No.	**30**	**31**	**32**
Structure			
pIC_{50}	P = 5.5, E = 5.8	P = 5.0, E = 5.6	P = 5.7, E = 5.0
No.	**33**	**34**	**35**
Structure			
pIC_{50}	P = 5.1, E = 5.2	P = 6.0, E = 5.8	P = 5.4, E = 5.8
No.	**36**	**37**	**38**	**39**
Structure				
pIC_{50}	P = 5.7, E = 6.0	P = 5.1, E = 5.9	P = 4.9, E = 4.5	P = 5.1, E = 4.4

Even though the inhibitors were developed specifically to occupy the steric regions at the N-terminal side, the C-terminal modifications also affected the activities. The model could distinguish weak C-terminal modifications (morpholinyl in inhibitors **27**, **38** and **39**) from the more potent ones (Ac-alanine in **29**), and this prediction was confirmed in the *in vitro* tests.

Study III is not the first QSAR study of SIRT1 inhibitors; a 3D QSAR study of small molecular SIRT1 inhibitors was included in one publication describing acridinediones as SIRT1 inhibitors (Alvala et al. 2012). There are, however, some concerns with the study of Alvala et al. Firstly, they used only 18 inhibitors with a narrow activity range: the inhibition activities cover only <0.8 log units. It is generally agreed that the end point values of QSAR studies should cover at least one log unit (Cherkasov et al. 2014). As if to emphasize this data homogeneity, half of the inhibitors in the study possessed the exactly same pIC_{50} values. Furthermore, the results of the QSAR study were not interpreted biologically or chemically in order to to facilitate inhibitor development (Cherkasov et al. 2014; Zhang et al. 2011). For the record, in study III the inhibitors covered a pIC_{50} range 2.2-6.6. The obtained CoMFA contours displayed good correspondence with the SIRT1 substrate binding site. In addition, the CoMFA model was used in development of new and potent SIRT1 inhibitors. In conclusion, the CoMFA model of substrate based SIRT1 inhibitors in study III overcomes some of the problems encountered when connecting biological activities with *in silico* activity predictions. Thus, it could be regarded as a scoring function designed individually in this case for sirtuin inhibitors.

5.3 VIRTUAL SCREENING OF NEW SIRTUIN INHIBITORS (IV)

In study III, the SIRT3 crystal structure bound with a stalled reaction intermediate (PDB: 3GLT) was used as the target in a virtual screening campaign (Jin et al. 2009). The substrate binding region of SIRT3 had been previously exploited in a virtual screening (Salo et al. 2013). Thus, to avoid simply obtaining similar results as obtained in that previous screening, the surface of SIRT3 was studied for other allosteric putative binding sites. A promising groove was found on the zinc binding domain and this was used as the target in the virtual screening. Initially, Glide HTVS was employed for the whole ZINC database and 5% of the best scoring poses were subsequently docked with Glide SP, from which the top 5000 poses were further refined with Glide XP. Out of these results, 1000 poses were visually inspected and six compounds were selected for *in vitro* testing. Three topics were evaluated in the visual selection of the compounds. Firstly, the compounds should possess chemical moieties not present in previously published sirtuin inhibitors. Secondly, glycosides and reactive groups, such as nitro, were avoided. Thirdly, the inhibitors should be available from their corresponding providers.

Out of the five compounds tested, three displayed >30% inhibition of SIRT3 at 200 μM. The poor solubility of one compound prevented its testing *in vitro*. To expand the SAR discussion of the manuscript, analogues of active compounds were searched from PubChem and ZINC database using Tanimoto similarity scoring with the >60% similarity. In total, 26 compounds were tested for their inhibitory activity against SIRT3. 15 compounds displayed >30% SIRT3 inhibition, and they were studied more closely.

Some of the studied compounds emitted fluorescence and were coloured in solutions, which could pose problems in a fluorescence based assay. To study the effect of the compounds on fluorescence, standard curves were determined for them with various concentrations of deacetylated standard. The deacetylated standard represents the assay substrate, which is already deacetylated and thus readily processed by trypsin to detach the fluorophore and produce fluorescence (see Figure 24). In the absence of other assay elements it is possible to study the effects of the compounds themselves on the fluorescence with increasing fluorophore concentrations (0 μM, 1.25 μM, 2.5 μM and 5 μM), which cover the observed fluorescence range of the sirtuin assays. Regression curves were determined for all the compounds individually

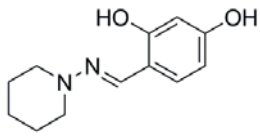
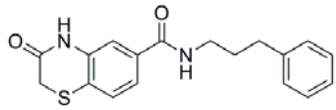
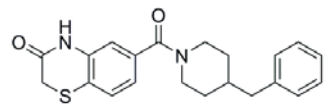
using the above deacetylated standard concentrations and the 200 μM inhibitor concentration used in the screening. When the regression curves were examined, it became evident that not all signal reduction observed with the compounds was caused by sirtuin inhibition. With the same amount of fluorophore some compounds displayed significantly reduced fluorescence compared to the undisturbed signal. The inhibition percentages of these compounds were exaggerated, since the observed signal reduction was partially caused by these artefactual properties of the compound and not sirtuin inhibition. Actual inhibition percentages were then calculated for all the compounds with Equation 2, which takes the signal interference into account:

$$inh\% = 1 - (S_{inh} - B_{inh})/(S_{DMSO} - B_{DMSO}) * (a_{DMSO}/a_{inh}) \qquad \text{Equation 2.}$$

In Equation 2 inh% represents actual sirtuin inhibition percentage. S_{inh} and S_{DMSO} refer to the signals observed in the assay with inhibitor and DMSO, respectively. B_{inh} and B_{DMSO} designate the background signals observed in the assay with inhibitor and DMSO, respectively. Finally, a_{inh} and a_{DMSO} the slopes of the regression lines obtained with the deacetylated standard. Most of the inhibitors displayed only poor (<30%) actual inhibition of SIRT3 according to Equation 2. However, for three compounds the actual inhibition matched their observed inhibitory activity, and their initial measurements were considered to be reliable (Table 5).

These three compounds inhibited the SIRT3 deacetylation by 30-40% at 200 μM concentration. To test their subtype specificity, the inhibition activities were also determined for SIRT1 and SIRT2 using the same concentration. All three inhibitors displayed only weak inhibition of SIRT1 (≤15%). Inhibitor **40** displayed 21% inhibition of SIRT2. However, compounds **41** and **42** with the bicyclic 3-oxo-1,4-benzothiazinyl scaffold displayed 60% and 40% respective inhibition of SIRT2 (Table 5). Even though **40** displayed the weakest inhibition against all tested sirtuins, its small size represents a useful starting point for future structural modifications. Inhibitor **40** had not been reported before as a biologically active compound, even though has been included in a patent application, which describes promising molecules to affect huntingtin degradation (Wanker et al. 2012). Inhibitors **41** and **42** have not been reported in any biological studies, but molecules with the same 3-oxo-1,4-benzothiazinyl scaffold have been tested for their antimalarial properties (Preuss et al. 2012). In addition, their relatively small size offers possibilities for structural modifications to increase both potency and subtype selectivity. Furthermore, since similar molecules have been tested for antimalarial properties, the available analogues for these compounds should be tested for their parasitic sirtuin inhibition.

Table 5. The inhibitory percentages of the identified inhibitors at 200 μM with SIRT1, SIRT2 and SIRT3. The mean and SD were calculated from at least three determinations.

N:o	**40**	**41**	**42**
Structure			
%±SD SIRT1	13 ± 0	15 ± 3	15 ± 3
%±SD SIRT2	21 ± 3	42 ± 2	60 ± 3
%±SD SIRT3	32 ± 4	40 ± 6	38 ± 1

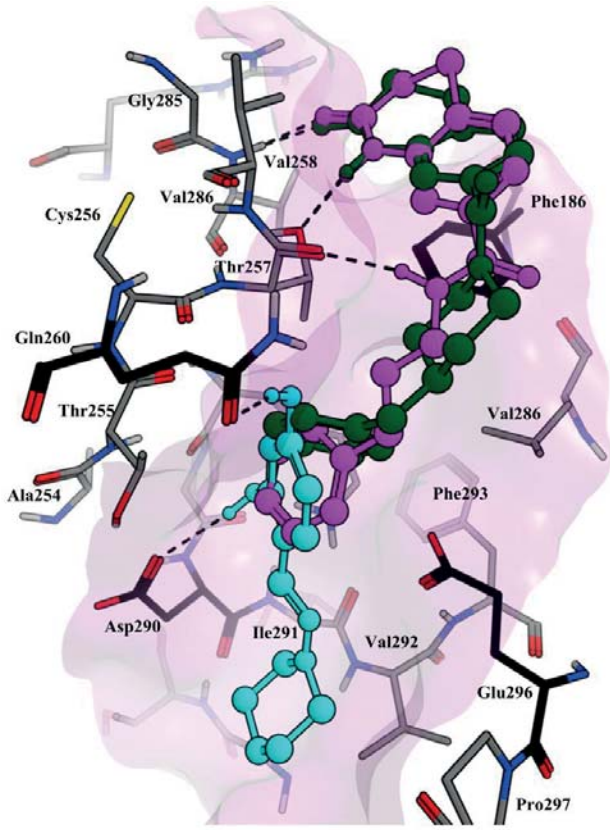


Figure 27. The interactions of the inhibitors **40** (cyan), **41** (magenta) and **42** (green) with the putative binding site of SIRT3. Residues coloured in black were used to define the GRID-file centre for the virtual screening. Reused with permission from Elsevier.

The interactions of inhibitors **40**-**42** with the putative binding site of SIRT3 are shown in Figure 27. Inhibitors **41** and **42** share a similar binding mode with hydrogen bonds to Val286 and Thr257. The smaller inhibitor **40** occupies another part of the putative binding site undertaking hydrogen bonding to Gln260 and Asp290. It is possible that more potent inhibitors could be designed by with a single compounds which could occupy the whole putative binding site and utilize all of the interaction possibilities. It is noteworthy, however, that the inhibitors might actually bind to other parts of sirtuins, but the exact binding sites and modes could only be determined if one could create the appropriate crystal structures.

As a conclusion, study IV produced three new sirtuin inhibitors with micromolar activities. The inhibitors display slight selectivity toward SIRT2-3 over SIRT1 and they have not been previously published as biologically active compounds.

6 General Discussion and Future Prospects

The present work utilizes many standard methods of drug discovery in studying sirtuin inhibitors. Combining the *in silico* and *in vitro* methods produced new information about sirtuin inhibition. However, some methodological features of the discovery of novel sirtuin inhibitors and the sirtuins themselves will be discussed here, but some aspects will need to be studied in more detail in the future.

Challenges of *in silico* methods

The intrinsically hypothetical nature of *in silico* methods is both their greatest asset and most severe drawback. The predictions emerging from biological binding are affinity be used to select only promising molecules for *in vitro* and *in vivo* testing, which reduces the costs and effort of the drug discovery projects. However, despite the giant leaps taken in molecular modelling during the last decades, the binding mode and especially affinity predictions many times fail.

Protein structures

Initially the problem with *in silico* methods was the lack of experimentally derived protein structures. Today X-ray crystallographic and computational methods can be combined to provide structural data for the majority of proteins, including sirtuins (Schwede 2013). The major weakness of the crystallographic structures is that they provide only a static representation of the dynamic protein under artificial conditions (Cheng et al. 2012). Like all proteins, sirtuins are highly flexible and their substrate binding induces conformational changes (see 2.2). In an attempt to model SIRT6 in a conformation suitable for docking of substrate based inhibitors, homology modelling was used in study I. The comparative models tend to resemble their template more than their target structure, which is seen also when comparing the obtained SIRT6 homology model (especially the zinc binding domain) and the crystallographically determined SIRT6 structure (MacCallum et al. 2011). Another problem encountered with the experimentally derived sirtuin structures is that they only represent the catalytic region, whereas the structures of the flanking C- and N-terminals remain unsolved. Furthermore, also the catalytic domain structures of SIRT4 and SIRT7 have not been solved. It is likely that their catalytic domain structures could be obtained by homology modelling, which was used in study I to derive the catalytically active conformation of SIRT6.

Molecular docking

The interactions of sirtuins and their inhibitors were studied with molecular docking in this thesis. Naturally, also the docking poses represent only a static image of the highly dynamic interaction between the protein and the inhibitor. The other major drawback with the docking of the small molecular sirtuin inhibitors is that there is only structural knowledge of individual binding modes of three inhibitors, and it is probable that not all small molecule inhibitors share the same binding site, let alone the binding mode. In this thesis and generally in molecular docking processes, the ligand flexibility is taken into account, but the protein flexibility is only partially used or neglected totally due to computational costs (Cheng et al. 2012). The problem of protein flexibility can be tackled with a generated set of protein conformations to create an ensemble to be used in the docking (Rueda et al. 2010; Sperandio et al. 2010). Because every conformation needs its own individual docking run, the method is computationally ineffective, and research is underway to overcome this problem (Fischer et al. 2014). Induced-fit docking is another method which can be applied to overcome this false rigidity of the protein, and it tries to mimic the conformational change of proteins occurring during and after ligand binding (Sherman

et al. 2006). The flexible docking would probably influence only slightly on the binding modes of the substrate based sirtuin inhibitors, since they are assumed to bind in a certain way. However, it could have an impact on the docking poses of the small molecular inhibitors, because the binding site could adjust itself individually for each compound.

Most of the interactions of the substrate based sirtuin inhibitors occur on the solvent accessible surface of sirtuins, and also the inhibitors in study IV are assumed to bind to the protein surface. However, the effects of solvation and desolvation in docking are often neglected, also in this thesis (Thilagavathi and Mancera 2010). The water molecules play a major role in ligand recognition, since they can form hydrogen bonds in the ligand-protein interface, especially on the surfaces of proteins. It is challenging to attempt to account forwater molecules in docking, since the presence or absence and orientation of water molecules depends on the individual ligands (Santos et al. 2010). Various methods have been devised to take the solvation effects into account during docking. They include the docking of ligands together with a bunch of water molecules and predicting the "wet and dry" regions of the binding site (Lie et al. 2011; Wang et al. 2011b). Ultimately, more research is needed to better incorporate the effect of water in molecular docking.

Exact docking scores of the inhibitors were not precisely reported in this thesis because of their hypothetical nature. The empirical scoring function, GlideScore, was used in all docking studies (Friesner et al. 2004). Since it is an empirical scoring function, GlideScore is derived from a large set of experimental ligand-protein complexes with the corresponding biological affinities. It is possible that this sort of generalization of ligand-protein interactions may not be the most advantageous route to take, but instead the scoring functions should be individually designed for each case (Seifert 2009). This was simulated in study III of this thesis, where the CoMFA model can be regarded as a scoring function specifically designed for substrate based SIRT1 inhibitors. Although scoring functions are often considered to lie at the heart of molecular docking, they still predict poorly the biological affinity of inhibitors. Many studies have explored the performance of various scoring functions, but a single scoring function has never outperformed all of the others in all cases (Cheng et al. 2012; Huang et al. 2010). In most cases the scoring functions can predict the correct binding pose for a ligand and distinguish between the active and inactive molecules, but accurate binding affinity predictions have yet to be devised. The new sirtuin inhibitors identified in study IV were found from high-scoring poses derived from virtual screening, which supports this claim. However, it is not certain that these inhibitors bind to the putative binding site of study IV, but instead it is possible that they bind to some other part of sirtuins. Only crystal structures would reveal the actual binding site with certainty.

In silico screening and commercial databases

Many sirtuin inhibitors have been found as a result of virtual screenings, such as those discovered in study IV. Despite almost ten years of hunting for inhibitors with sirtuin crystal structures and/or homology models, rather few sirtuin inhibitors with nanomolar potencies have been found. Instead, many mediocre sirtuin inhibitors with micromolar activity have been published, and the subsequent medicinal chemistry efforts to increase the selectivity and/or potency of these initial hits have failed. It is possible that the chemical spaces of commercial screening databases are biased towards compounds targeting "druggable" proteins, such as G-protein coupled receptors and kinases, and thus they may not incorporate the chemical space of potent sirtuin inhibitors (Tsukamoto 2013). In addition, the databases are somewhat homogenous in their compounds, since almost 1300 ring structures present in natural products cannot be found in commercial databases (Hert et al. 2009). The natural molecules containing these ring structures have evolved to interact with at least one protein in an organism. This indicates that they have at least some desirable properties which could be exploited in the search for new drugs, even if it is only solubility and cell permeability. However, though using natural product libraries in screening might provide useful hits for challenging targets, such as sirtuins, the synthetic production of these kinds of molecules might prove rather difficult (Bauer et al. 2010). Nevertheless, based on the unimpressive sirtuin inhibitors discovered by virtual screening, one

must conclude that either the current databases do not contain truly potent sirtuin inhibitors or the current *in silico* screening methods are not able to distinguish these interesting compounds from their less useful counterparts.

3D QSAR

3D QSAR was successfully used in this thesis for the design of new substrate based SIRT1 inhibitors. The CoMFA method used here, like many other 3D QSAR methods, neglects the flexibility of ligands and possibility that there may be many biologically active conformations, because only one pose of each compound is used. If one wants to remove the need for alignment, there are alignment-free descriptors available, such as GRID independent descriptors (GRIND2) (Duran et al. 2008). However, since the binding pose of substrate based sirtuin inhibitors is known, the alignment of inhibitors to this structure should produce more relevant data than data obtained from the inhibitor structures alone. Nowadays the 3D QSAR methods are slowly being replaced by more advanced proteochemometric studies (Cortes-Ciriano et al. 2014; Wikberg et al. 2003; Willighagen et al. 2011). In proteochemometrics, the biological activities are not studied only in the context of a single protein target, but multiple targets can be used. This would be extremely interesting in the case of sirtuin inhibitors, as the compounds generally display at least some degree of subtype selectivity. Proteochemometrics could be utilized to distinguish the features that contribute to the selectivity. However, no comprehensive inhibitor screenings have been conducted for all sirtuins and most importantly, the binding sites and modes of most inhibitors remain elusive.

Challenges of *in vitro* methods

Even though the determination of *in vitro* activity of new compounds is a crucial step in drug design, rather few practical methods have been developed to assess sirtuin activity. The first sirtuin assays were based on the radioactive substrates followed by separation of labelled reaction products and scintillation counting (Borra and Denu 2004; McDonagh et al. 2005). The other described methods included enzyme-linked immunosorbent (ELISA), capillary electrophoresis, magnetic beads based chromatography, mass spectrometry and bioluminescence assays (Fan et al. 2008; Fan et al. 2009; Liu et al. 2008; Mahajan et al. 2011; Schlicker et al. 2008; Yasuda et al. 2011). Despite the possibilities offered by the different assays, the research field has been dominated by assays based upon fluorescence substrates, which are relatively cost-effective and scalable up to industrial level. In those assays, the substrate contains a fluorophore, which is cleaved off after sirtuin deacetylation with another enzyme, such as trypsin (see 4.2 and Figure 24) (Heltweg et al. 2003; Marcotte et al. 2004; Wegener et al. 2003).

The *in vitro* sirtuin assays might be another cause to the scarcity of nanomolar sirtuin inhibitors. It could be that *in vitro* sirtuin activity assays cannot differentiate truly potent inhibitors from each other because of the assay reliability limits. The *in vitro* assays display only the function of the recombinant enzyme under artificial conditions, which might not mimick the situation in cells with sufficient accuracy.

Signal interference

All the *in vitro* data of this thesis is derived from *in vitro* sirtuin assays, which are based on the differences of the measured emitted fluorescence, so it is not unreasonable to assume that fluorescent compounds can cause problems with this kind of assay (Thorne et al. 2010). Around 2-5% of compounds in a chemical library contained heterocyclic and conjugated moieties, which emit fluorescence with the excitation and emission wavelengths of the blue spectral region used in the sirtuin assays (Simeonov et al. 2008). Furthermore, in the same study it was concluded that by using blue spectral excitation and emission wavelengths up to 50% of the identified active molecules were fluorescent. The problems of fluorescent compounds can be minimized by including a time-delay after excitation or by changing the fluorophore (Thorne et al. 2010). For example, less than 0.01% of compounds displayed fluorescence at the longer, orange spectra

excitation and emission wavelengths of resorufin (Simeonov et al. 2008). Unfortunately, unlike AMC, resorufin cannot be as easily linked to fluorogenic assay substrates and it has not been exploited in any sirtuin assays.

Another factor related to the physicochemical properties of the compounds which causes disturbances in fluorescent assays is their colour. Colourful compounds can absorb light in the wavelengths used in the assay, which can over-estimate their inhibition activity. In sirtuin assays this is mostly caused by yellow and orange compounds, because they are the complemental colours which absorb the ultraviolet-blue spectral wavelengths used in the assay. Furthermore, compounds can be both fluorescent and colourful, and in these cases it might be difficult to determine the combinational effect of these two properties on the observed inhibition. In study IV, these effects studied with the deacetylated standard, which is provided in the commercial assay kit. By determining the effect of the compounds to the observed fluorescence, their actual sirtuin inhibition could be approximated. However, it is not usual to be sure that the deacetylated standard or other signal interference determination methods have been included in manuscripts describing sirtuin inhibitors, which leaves one to speculate whether the authors have even considered the possibility of signal interference.

Perhaps the simplest solution to combat signal interference is to apply a combination of different *in vitro* methods (Mahajan et al. 2011). The combination of two different assays would have helped to identify the signal interference inducing compounds in study IV. Thus, the fluorescence based assays could be applied as fast and efficient method for screening new hits, and then other techniques, such as mass spectrometry, could be used in the determination of the IC_{50} values. The advantage of utilizing two or more methodologically different assays in inhibitor screening would reduce the risk of stumbling into signal interference. However, this naturally increases the cost of screening. It seems obvious that this research field desperately needs an efficient and fast screening method, where fluorescent and colourful compounds do not introduce spurious problems.

Frequent hitters

Even though the new sirtuin inhibitors discovered in study IV had not previously been reported as biologically active compounds, the issue of frequent hitters is a problem also in sirtuin inhibitor screening. Compound aggregation, reactivity and possible interference with reaction components other than the enzyme can cause problems in all enzymatic assays (Thorne et al. 2010). There are many known structural moieties, which are present in frequent hitters or promiscuous compounds that show up in many different kinds of *in vitro* assays (Baell and Holloway 2010; Schorpp et al. 2013). Even though many of the databases are filtered in order to remove the most reactive compounds, many structural features are increasingly more prevalent in the literature due to their highly promiscuous nature (Baell and Holloway 2010). Even though many marketed drugs have multiple biological targets, it is a matter of debate whether it is advisable to pursue polypharmacology already at the preclinical stage of drug design (Boran and Iyengar 2010). Quercetin is an extreme example of a promiscuous compound; it was also noted in study II to inhibit the deacetylation reaction of SIRT6. According to PubChem, quercetin has been determined as being active in more than 25% of the biological assays reported. Quercetin forms large aggregates in the aqueous environment and is considered as a highly condition-sensitive inhibitor of various proteins, which makes it a poor lead molecule in drug development (McGovern and Shoichet 2003; Pohjala and Tammela 2012).

SIRT6 deacetylation assay

Even though the *in vitro* deacetylation activity assays have been successfully developed and are continuously used in screening of SIRT1-3 inhibitors, it seems that such a simple approach is not so appropriate for SIRT6. The specific deacetylation activity of SIRT6 causes problems with the fluorogenic assay substrates, even though it was shown in study II that the assay substrate based on H3K56 was more rapidly catalysed than that based on p53. It is tempting to think that the

H3K56 based assay substrate is more natural than that based on p53, but the linked fluorophore still makes it an artificial substrate. The high deacetylation specificity of SIRT6 could be mediated by the C-terminal residues after the N^{ε}-Ac-lysine, since the H3K9 and H3K56 both share the sequence Lys(Ac)-Ser-Thr. These residues cannot be taken into consideration in the development of this type of fluorogenic assay substrates, because the fluorophore must be directly linked to the carboxyl terminal of the N^{ε}-Ac-lysine in order to allow cleave by trypsin (Wegener et al. 2003). Furthermore, the fluorogenic assay substrate used in study II is short and it contains only four residues. It is possible that the specific SIRT6 deacetylation reaction is dependent on the contact with a longer peptide chain or some other parts of the substrate.

The addition of free fatty acids and albumin could not enhance the deacetylation activity of SIRT6 *in vitro* in the assay applied in study II. It is possible that there is some other regulatory protein or cellular marker, which is crucial for SIRT6 deacetylation activity but which has not been discovered yet. It is also possible that the recombinant human SIRT6 produced in bacteria is not folded properly or lacks important post-translational modifications, which causes it to display poorer deacetylation activity *in vitro* than *in vivo*. During the initial determination of optimal assay conditions in study II, it was observed that the recombinant SIRT6 was extremely sensitive to solvent concentration and freezing-thawing cycles. There might be some property inherent in the SIRT6 protein or within the assay conditions, which make it more unstable than other sirtuins *in vitro*.

SIRT6 differs from SIRT1-3 because it has many different catalytic activities. In this thesis, only the deacetylation activity of SIRT6 was taken into account. It will be fascinating to see if the other different catalytic activities of SIRT6 are can be exploited in the future. For example, will it be possible to study the overall effect of inhibitors on SIRT6 in a single assay, or will it be mandatory to use multiple assays with different substrates to monitor the effects of inhibitors individually on each catalytic activity?

Sirtuin inhibitors

All sirtuins are involved in many different biological pathways and functions, which might be problematic for inhibitor design and assay development. The inhibition of certain sirtuin pathways could lead to adverse effects, and sirtuin subtype specificity might not be sufficient to avoid this potential problem. It may be desirable to target only a certain sirtuin-substrate pathway, and if so, inhibitors will need to be designed to selectively affect only one single protein-protein interaction. Even if it were possible to develop such a specific molecule, it remains unclear if the inhibition would be sufficient to exert meaningful a pharmacological effects *in vivo*. For example, the changes evoked by the inhibitor might be negated by changes in the activities or functions of other proteins and enzymes.

It is evident that sirtuins could be targeted by drug molecules, which would either increase or decrease their activity. It is also possible that sirtuin activity could be regulated with indirect routes, such as targeting NAD^+ production or by affecting the activities of enzymes that post-translationally modify sirtuins. The advances in sirtuin inhibitor design and discovery have made sure that sirtuins will remain in focus of drug design for years to come. Developing a new reliable *in vitro* screening method and combining it with the increasing structural information with *in silico* methods could lead to a pharmacological revolution of sirtuin targeting drugs.

Thio-Ac-lysine containing inhibitors

From the drug design perspective, the first N^{ε}-Ac-lysine containing peptidic sirtuin inhibitors exhibited the general problems of peptides, such as poor water solubility and degradation (Fosgerau and Hoffmann 2014). Shortening the peptide chains and implementing artificial moieties instead of natural amino acids in pseudopeptides has produced more "drug-like" inhibitors of sirtuins. In particular, the revelation of thio-Ac-lysine has contributed to potent and cell penetrable inhibitors of sirtuins, some of which were also designed in study III (Mellini et al. 2013; Suzuki et al. 2009). Even though these kinds of inhibitors can be useful tools in elucidating

sirtuin function at the cellular level, their development to drugs is hindered by the intrinsic toxicity of the thioamide moiety and its metabolites (Kang et al. 2008; Neal and Halpert 1982; Ruse and Waring 1991). The *S*-oxidation of thioamides produces the corresponding sulfoxides and *S,S*-dioxides, which are especially toxic to hepatocytes due to their covalent bonding capabilities (Chilakapati et al. 2007; Kang et al. 2008). However, it is possible, that the problems of the metabolites could be avoided by using different administration modes instead of the oral route (Houston and Taylor 1984).

7 Conclusions

It is concluded that:

1. Some sirtuin inhibitors affect the deacetylation activity of SIRT6. Thus, SIRT6 inhibition should not be overlooked when testing subtype specificity of sirtuin inhibitors.

2. SIRT6 deacetylates more efficiently an assay substrate based on its natural substrate H3K56 than one based on a non-natural substrate of SIRT6, p53. This new assay substrate can be used in the screening of SIRT6 inhibitors.

3. A 3D QSAR model of substrate based inhibitors can be used to design new SIRT1 inhibitors. The corresponding CoMFA contours are in good agreement with the SIRT1 substrate binding region, which supports the biological plausibility of the model.

4. A putative binding site on the zinc binding domain of SIRT3 was successfully used in virtual screening of sirtuin inhibitors. Three moderately potent inhibitors were found; they contain structures not present in previously published sirtuin inhibitors and they displayed slight selectivity toward SIRT2-3 over SIRT1.

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Piia Kokkonen

Studying Sirtuin Inhibitors with In Silico and In Vitro Approaches

Sirtuins are a group of epigenetic enzymes, which work in the interface between metabolism, gene expression and protein function. Even though much attention is paid for the activation of sirtuins with compounds from red wine and plants, the inhibition of sirtuins also offers interesting pharmacological possibilities in treatment of cancer and metabolic diseases. In this thesis, sirtuin inhibitors were studied with in silico and in vitro methods. The results included new sirtuin inhibitors and insights into their subtype specificity.

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