



## Quercetin based derivatives as sirtuin inhibitors

Vladimír Heger<sup>a,1</sup>, Jonna Tyni<sup>b,1</sup>, Attila Hunyadi<sup>c</sup>, Lubica Horáková<sup>a</sup>, Maija Lahtela-Kakkonen<sup>b</sup>, Minna Rahnasto-Rilla<sup>b,\*</sup>

<sup>a</sup> Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine SAS, Dúbravská 9, 84104, Bratislava, Slovakia

<sup>b</sup> University of Eastern Finland, School of Pharmacy, P.O. Box 1627, 70210, Kuopio, Finland

<sup>c</sup> Institute of Pharmacognosy, Interdisciplinary Excellence Centre, University of Szeged, Eötvös u. 6, 6720, Szeged, Hungary



### ARTICLE INFO

#### Keywords:

Sirtuin  
SIRT6 inhibitor  
SIRT2 inhibitor  
Quercetin derivatives  
Deacetylation

### ABSTRACT

Polyphenols synthesized by plants and fungi have various pharmacological effects. The ability of polyphenols to modulate sirtuins has gained considerable interest due to the role of sirtuins in aging, insulin sensitivity, lipid metabolism, inflammation, and cancer. In particular, sirtuin 6 (SIRT6) has gained importance in regulating a variety of cellular processes, including genomic stability and glucose metabolism. On the other hand, quercetin has been demonstrated to modulate sirtuins and to protect against several chronic diseases. In this study, two quercetin derivatives, diquercetin and 2-chloro-1,4-naphthoquinone-quercetin, were identified as promising SIRT6 inhibitors with  $IC_{50}$  values of 130  $\mu$ M and 55  $\mu$ M, respectively. 2-Chloro-1,4-naphthoquinone-quercetin also showed potent inhibition against SIRT2, with an  $IC_{50}$  value of 14  $\mu$ M. Diquercetin increased the  $K_m$  value of  $NAD^+$ , whereas 2-chloro-1,4-naphthoquinone-quercetin increased the  $K_m$  value of the acetylated substrate. Molecular docking studies suggest that diquercetin prefers the binding site of the nicotinamide (NAM) moiety, whereas 2-chloro-1,4-naphthoquinone-quercetin prefers to dock into the substrate binding site. Overall, the results of *in vitro* studies and molecular modeling indicate that diquercetin competes with nicotinamide adenine dinucleotide ( $NAD^+$ ), whereas 2-chloro-1,4-naphthoquinone-quercetin competes with the acetylated substrate in the catalytic site of SIRT6. Natural polyphenolic compounds targeting sirtuins show promise as a new approach in the search for novel and effective treatments for age-related diseases.

### 1. Introduction

Sirtuins (SIRT) are  $NAD^+$ -dependent protein deacetylases that have emerged as exciting targets for several diseases. There are seven SIRT in mammals that differ in cellular sublocation: SIRT1, SIRT6, and SIRT7 are nuclear enzymes, SIRT2 is primarily cytosolic, and SIRT3, SIRT4, and SIRT5 are found in mitochondria. The regulation of SIRT has been studied over the past 15 years and has led to the identification of many SIRT inhibitors and activators. Most of these compounds target SIRT1, but more regulators have recently been identified for other SIRTs.

SIRT6 plays an important role in DNA damage signaling and repair and is involved in metabolism; therefore, it is a potential therapeutic target in the context of neurodegenerative diseases, metabolic disorders

including diabetes, and cancer [1–3]. Several studies indicate that SIRT6 levels are reduced in some types of cancers and that SIRT6 deficiency results in genetic instability and tumorigenesis [4,5]. However, some reports have shown that SIRT6 may play an opposite role and act as a tumor promoter. For example, SIRT6 is upregulated in some cancers, such as hepatocellular carcinoma and multiple myeloma, and the overexpression of SIRT6 is associated with poor prognosis [6]. SIRT6 also influences metabolic pathways, such as glycolysis and gluconeogenesis, which may affect tumor growth. Taken together, these findings indicate that SIRT6 function is associated with multiple key pathways related to aging. However, the medical and therapeutic relevance of SIRT6 in humans remains incompletely understood.

Black mulberry (*Morus nigra*) is rich in polyphenols and alkaloids, which have hypoglycemic, hypolipidemic, hypotensive, anti-

**Abbreviation:** ADP, adenosine diphosphate; ALR2, aldose reductase; ATP, adenosine triphosphate; DMSO, dimethyl sulfoxide; ELC, enhanced chemiluminescence; HDAC, histone deacetylase; H3, histone 3; H3K9, histone 3 lysine 9; H3H56, histone 3 lysine 56; HRP, horseradish peroxidase; NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide; NMR, nuclear magnetic resonance; PDB, protein data bank; PVDF, polyvinylidene difluoride; SERCA, sarcoplasmic reticulum  $Ca^{2+}$ -ATPase; SirReal2, sirtuin-rearranging ligand2; SIRTs, sirtuins

\* Corresponding author.

E-mail address: [Minna.Rahnasto@uef.fi](mailto:Minna.Rahnasto@uef.fi) (M. Rahnasto-Rilla).

<sup>1</sup> Equal contribution.

<https://doi.org/10.1016/j.bioph.2019.01.035>

Received 18 October 2018; Received in revised form 8 January 2019; Accepted 8 January 2019

0753-3322/ © 2019 The Authors. Published by Elsevier Masson SAS. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

inflammatory, and anti-tumor effects [7–9]. Quercetin is one of the most studied polyphenols and it has many health benefits. However, its low bioavailability prevents clinical use and thus there is need for developing derivatives with better pharmaceutical properties. Novel quercetin derivatives, mainly with pivaloyl-, acetyl-, feruloyl-, and caffeoyl-groups, have been previously designed, synthesized, and characterized with respect to antioxidant/pro-oxidant, cytotoxic, anti-tumor, and anti-inflammatory properties [10,11]. The quercetin derivatives that have an inhibitory effect on aldose reductase and alpha-glucosidase may be promising in the treatment of diabetic disorders [12,13]. Interestingly, quercetin has been shown both *in vitro* and *in vivo* SIRT1 activation either directly or indirectly [14,15]. Quercetin together with resveratrol upregulates SIRT1 and SIRT2 expression *in vivo* [16]. The role of quercetin is not restricted to SIRT1. We have previously shown that polyphenols like kaempferol and quercetin exceed 40% SIRT6 inhibition at 10  $\mu$ M and 100  $\mu$ M concentrations, respectively [17].

Novel quercetin derivatives, mainly pivalate or halogen-bearing compounds, have also been shown to induce a significant concentration-dependent decrease in sarcoplasmic reticulum  $\text{Ca}^{2+}$ -adenosine triphosphatase (ATPase) (SERCA) activity. SERCA maintains  $\text{Ca}^{2+}$  homeostasis, and alteration in its expression and activity can result in myopathy, heart failure, diabetes, or cellular malignancy. The anticancer properties of flavonoids may be mediated by their ability to induce apoptosis via the  $\text{Ca}^{2+}$ -dependent mitochondrial pathway [18] and by inhibiting SERCA [19]. On the other hand, some quercetin derivatives stimulate SERCA activity. Modulation of SERCA by polyphenols may represent an approach to the therapy of diseases involving SERCA impairment [20]. One example of such diseases is diabetes, in which there are disruptions in  $\text{Ca}^{2+}$  homeostasis affects pancreatic insulin and glucagon secretion. Interestingly, SIRT6 is also linked to maintaining  $\text{Ca}^{2+}$  homeostasis as it regulates the production of  $\text{Ca}^{2+}$ -mobilizing nucleotides [21].

The aim of this study was to identify novel types of scaffolds for designing potent SIRT6 inhibitors because there are still a limited number of SIRT6 inhibitors. We explored how a set of quercetin and rutin derivatives, along with other polyphenols isolated from the root bark of *Morus nigra*, affect SIRT6 deacetylation activity. Previously these compounds were shown to have the ability to modulate SERCA activity and have promising cytotoxic, anti-cancerous, and anti-inflammatory properties [22–25]. We observed that the most potent inhibitors of SIRT6 were diquercetin (compound 25) and 2-chloro-1,4-naphthoquinone-quercetin (compound 3). We also studied the possible binding poses and interactions of these inhibitors with molecular docking. Interestingly, they also showed inhibition towards other sirtuins.

## 2. Materials and methods

### 2.1. Polyphenols

Kaempferol, quercetin and rutin (compound 11) were ordered from Sigma Aldrich (USA). Quercetin derivatives (compounds 2, 3, 7, 8, and 20–25) were synthesized as described previously by Veverka et al. [10], and compound 12 was synthesized according to Raza et al. [26]. Rutin derivatives were prepared via lipase-catalyzed esterification of rutin with palmitic (compound 18), stearic (compound 19), or arachidonic acid (compound 14) in 2-methylbutan-2-ol at 60 °C as previously published [27]. Pycnogenol (compound 13) is from Horphag Research Ltd. (UK). Polyphenolic compounds from the root bark of *Morus nigra* were isolated in pure form and identified by high-resolution mass spectrometry (HRMS) and 2D nuclear magnetic resonance (NMR) techniques: morusin (compound 4) according to Kim et al. [28]; kuwanon E (compound 5) and kuwanon U (compound 6) according to Jeong et al. [29]; moracin P (compound 9) as described by Lee et al. [30]; and moracin R (compound 10) as described by Kapche et al. [31].

Benzothioephene derivatives (compounds 15, 16, 17) were synthesized according to Alsgo et al. [32,33].

### 2.2. Materials

7-Amino-4-methylcoumarin (AMC) labelled substrate, Ac-RYQK (Ac)-AMC, was purchased from CASLO ApS (Denmark).  $\text{NAD}^{+}$  was from Sigma Aldrich (USA). Enhanced chemiluminescence (ECL) prime western blotting detection reagents were from Amersham BioSciences (UK). Novex 10–20% gradient gels and anti-rabbit-IgG (mouse) Horseradish peroxidase (HRP)-conjugated secondary antibody (G21234) were from Life Technologies (UK). Rabbit anti-acetyl histone 3 lysine 9 (H3K9) antibody (06-942) and purified chicken core histones (13–107) were from Millipore (USA). Rabbit anti-histone 3 (H3) antibody (9715S) was purchased from Cell Signaling Technology (USA).

Human SIRT6 expression vector hSIRT6-pGEX-6P3 was kindly provided by Prof. Katrin Chua (Stanford, USA). Recombinant GST-tagged SIRT6 was produced by fermentation in *E. coli* BL21(DE3)-pRARE. The production was done at + 16 °C with 0.1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) for 20 h and the soluble over-expressed protein was purified on glutathione agarose (Sigma, Saint Louis, USA).

### 2.3. *In vitro* assays for SIRT6

#### 2.3.1. SIRT6 screening

At first a set of compounds together with positive controls kaempferol and quercetin, were screened against SIRT6 using deacetylation assay as previously described [34]. Experiments were replicated three times, except for compounds 12 and 14 that were measured two times, and the data were expressed as mean  $\pm$  standard deviation (SD). Briefly, compounds in dimethyl sulfoxide (DMSO) (200  $\mu$ M), the final solvent concentrations in the samples did not exceed 3%, were incubated for 90 min with GST-SIRT6 (4.5  $\mu$ g/well), Ac-RYQK(Ac)-AMC (320  $\mu$ M), and  $\text{NAD}^{+}$  in Tris Buffer [25 mM, pH 8.0] at + 37 °C. The sample reactions were terminated by adding 50  $\mu$ l of developer solution (6  $\mu$ g/ $\mu$ l trypsin and 40 mM NAM) to all wells and followed by addition of SIRT6 to the control wells. The plates were incubated for 30 min at room temperature and the fluorescence was measured with excitation and emission wavelengths of 380 and 440 nm, respectively, using En-Vision 2104 Multilabel Reader (PerkinElmer, Finland).

**2.3.1.1. Immunoblotting assay.** Screening assay results of the most potent compounds, as well as those of positive controls, kaempferol and quercetin, were confirmed using western blot analysis as previously described [35]. Briefly, 50  $\mu$ M and 200  $\mu$ M concentrations of compounds and 2.5% DMSO control were incubated for 30 min in the presence of 3  $\mu$ g of purified recombinant GST-SIRT6, 1.25  $\mu$ g purified chicken core histones, and 500  $\mu$ M  $\text{NAD}^{+}$  in 25 mM Tris-HCl, pH 8.0 at + 37 °C. The reaction was stopped with Laemmli (sample buffer) and proteins were separated by SDS-PAGE using 10–20% gradient gels and transferred onto polyvinylidene difluoride (PVDF) membranes. H3K9 acetylation was detected with rabbit anti-acetyl H3K9 antibody (1:2000) followed by anti-rabbit HRP-conjugated secondary antibody (1:10 000). Membranes were stripped and re-probed with rabbit anti-histone H3 antibody (1:8000). Chemiluminescent signal detection and image acquisition were carried out using ECL prime western blotting detection reagents. Densitometric analysis of protein bands was carried out using ImageJ 1.32 software and followed by statistical analysis using Graph Pad Prism Software version 6 (California, USA).

#### 2.3.2. Dose response and Lineweaver-Burk plots analysis

Dose responses of the most potent compounds (25 and 3) were determined at a concentration range of 12  $\mu$ M to 1000  $\mu$ M as described above (2.3.1) as means  $\pm$  SD from triplicate determinations. Percentages of remaining SIRT6 activity were plotted as a function of

the logarithm of compounds' (25 and 3) molarity, and the curves were fitted to a sigmoid dose-response equation using Graph Pad Prism Software version 6 (California, USA). Lineweaver-Burk plots (Double-reciprocal plots) assay was performed as described above (2.3.1) in the presence or absence of compounds 25 or 3 (200  $\mu$ M, final concentration). Samples contained a constant concentration of NAD<sup>+</sup> (3 mM) or peptide substrate (600  $\mu$ M) with increasing concentrations of peptide substrate (ranging from 100  $\mu$ M to 1.2 mM) or NAD<sup>+</sup> (ranging from 300  $\mu$ M to 2.4 mM). Lineweaver-Burk plots analyses were constructed as means  $\pm$  SD from three different experiments using Graph Pad Prism Software version 6 (California, USA).

#### 2.4. In vitro studies for SIRT1–3

To investigate the activity of the most potent compounds (25 and 3) towards other sirtuins, the deacetylation activities of SIRT1–3 were determined as previously described with a Fluor de Lys sirtuin assays ( $n = 3 \pm$  SD). Since 200  $\mu$ M concentration of compound 3 resulted in robust inhibition of the other sirtuins, a lower concentration (50  $\mu$ M) was used to study variation between different SIRTs. Since compounds 25 and 3 showed good inhibition towards SIRT2, lower concentration of compounds (10  $\mu$ M) were also examined, and known sirtuin inhibitors Ex-527 and Suramin was included as reference compounds.

Assays based on the method described in the BioMol product sheet (Enzo Life Sciences, Ann Arbor, MI, USA). BioMol KI177 substrate for SIRT1 and KI179 substrate for SIRT2 and SIRT3 were used. Briefly, the reaction was started by incubating the enzyme (SIRT1, SIRT2 or SIRT3) with the reaction mixture containing acetylated peptide substrate (0.7 Km: 58  $\mu$ M for SIRT1, 198  $\mu$ M for SIRT2, and 32  $\mu$ M for SIRT3), NAD<sup>+</sup> (0.9 Km: 558  $\mu$ M for SIRT1, 547  $\mu$ M for SIRT2, and 2 mM for SIRT3), and 5% DMSO or compounds in 5% final DMSO concentration. Incubation was done at 37 °C, and lasted for 1 h. The developer and nicotinamide (2 mM in histone deacetylase (HDAC) assay buffer giving total volume of 50  $\mu$ l) were added and the incubation was continued for 45 min at 37 °C. The fluorescence was determined with excitation and emission wavelengths of 355 nm and 460 nm respectively using a Victor™ 1420 Multilabel Counter (PerkinElmer Inc., Waltham, MA, USA). Alternatively, the fluorescence was measured using EnVision 2104 Multilabel Reader (PerkinElmer, Waltham, MA, USA) with excitation and emission wavelengths of 370 nm and 460 nm, respectively.

#### 2.5. Molecular modeling methods

Schrödinger Maestro software version 11.4.011 (Small-Molecule Drug Discovery Suite 2017-4) [36] was used in molecular modeling studies. The most potent compounds, 25 and 3 were constructed and prepared with LigPrep at pH 7.4 using standard settings. Protein structures of SIRT6 (protein data bank (PDB) ID 3ZG6) [37] and SIRT2 (PDB ID: 4RMG) [38] were preprocessed with standard settings at pH 7.4 using protein Preparation Wizard. All possible isomers for compound 25 were constructed due to the fact that a mixture of compound 25 isomers was applied in *in vitro* testing.

Two grids for SIRT6 were constructed. The center of the first grid was in the middle of residues Leu7, Pro9, Ala11, Gly50, Phe62, Phe84, Ser86, and Asp114. This grid site is close to the NAM moiety binding site of NAD<sup>+</sup>, which is a quite common inhibitor binding site among other sirtuins. The center of the second grid was at substrate binding site in the middle of the residues Val113, His131, and Leu184. The grid center for SIRT2 was set to be in the middle of co-crystallized ligand, sirtuin-rearranging ligand2 (SirReal2). Compounds were docked with InducedFit Docking and with standard settings.

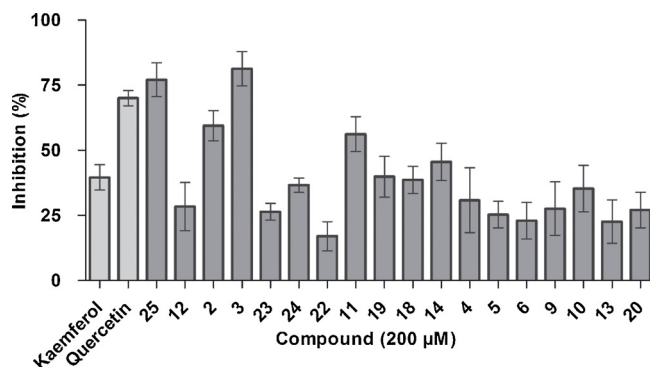


Fig. 1. Compounds 25, 2, 3, and 11 showed over 50% inhibition towards SIRT6 at 200  $\mu$ M concentration. Light grey bars represent inhibition of reference compounds, kaempferol and quercetin. The data are presented as mean  $\pm$  SD, ( $n = 3$ ), ( $n = 2$  for compounds 12 and 14) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

### 3. Results

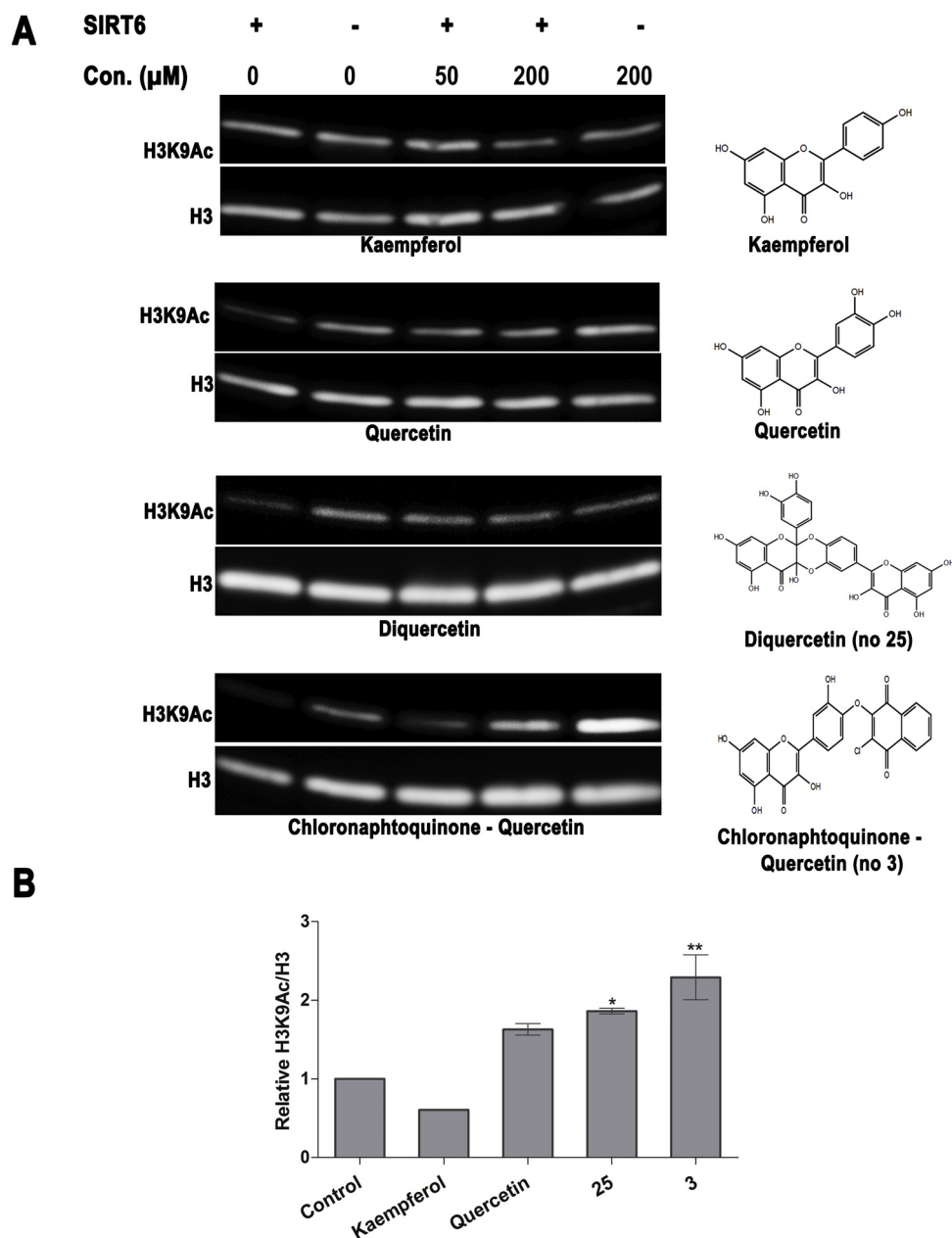
#### 3.1. In vitro studies for SIRT6

A set of polyphenols (see Supplementary Table S1) was investigated for SIRT6 inhibition using a fluorogenic-based deacetylation assay at a concentration of 200  $\mu$ M (Fig. 1). Known SIRT6 inhibitors, kaempferol and quercetin, were used as reference compounds and showed 38% and 68% inhibition of SIRT6, respectively. We were interested in determining those compounds that exceeded 50% inhibition of SIRT6.

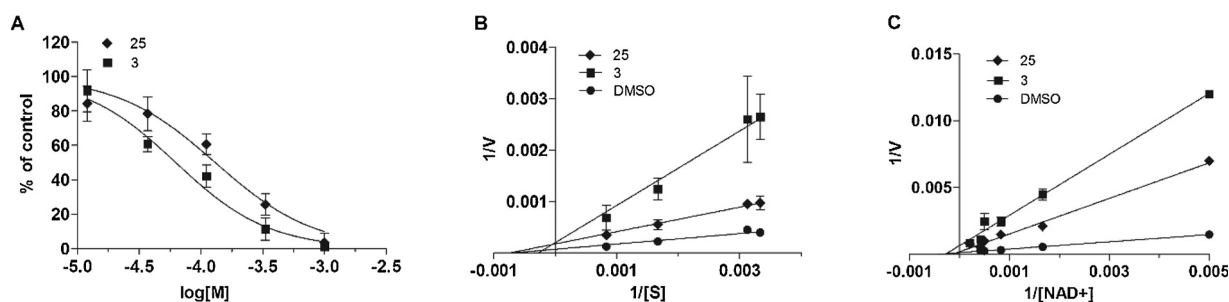
Interestingly, the most potent inhibitors were diquercetin (compound 25) and 2-chloro-1,4-naphthoquinone-quercetin (compound 3), which exceeded 80% inhibition. In contrast, the combination of three quercetin molecules bound with Fe-salt (compound 12) had an inhibition potency below 40%. Compound 2 showed 60% inhibition, whereas the rest of the quercetin derivatives were weak inhibitors. Rutin (compound 11) showed inhibition of 60%, but rutin derivatives exhibited only 40% inhibition.

The inhibitory effect against SIRT6 deacetylation activity of the known SIRT6 modulators and the most potent compounds of this study were confirmed using *in vitro* immunoblotting assay with core histone protein 3 as a substrate (Fig. 2, Supplementary Fig. S1). The core histone consisted of H2A, H2B, H3, and H4 proteins. The remaining levels of H3 acetylation on lysine 9 were determined to evaluate inhibition of compounds. H3K9Ac levels were normalized relative to H3, and quantification was presented as fold of change with respect to the control. The results with core histone show that kaempferol stimulated SIRT6 deacetylation activity at a concentration of 200  $\mu$ M. In contrast, quercetin decreased activity at both concentrations although not significantly. Like the results using a fluorogenic-based assay, the core histone studies showed that compounds 25 and 3 inhibited SIRT6 at a concentration of 200  $\mu$ M.

We also performed a dose-response study for the most potent compounds, compounds 25 and 3, which displayed IC<sub>50</sub> values of 130  $\mu$ M and 55  $\mu$ M, respectively (Fig. 3A). There was no obvious variation in the inhibitory activity of compound 25 with different peptide substrate concentrations, suggesting that compound 25 is not a substrate competitive inhibitor (Fig. 3B). In contrast, compound 25 demonstrated differences in inhibition with varying NAD<sup>+</sup> cofactor concentrations and, as a result, an increased Km value indicating cofactor competitive inhibition (Fig. 3C). Interestingly, compound 3 increased the Km value of substrate and inhibited SIRT6 in a competitive manner (Fig. 3B), whereas no competition against NAD<sup>+</sup> cofactor was observed (Fig. 3C).

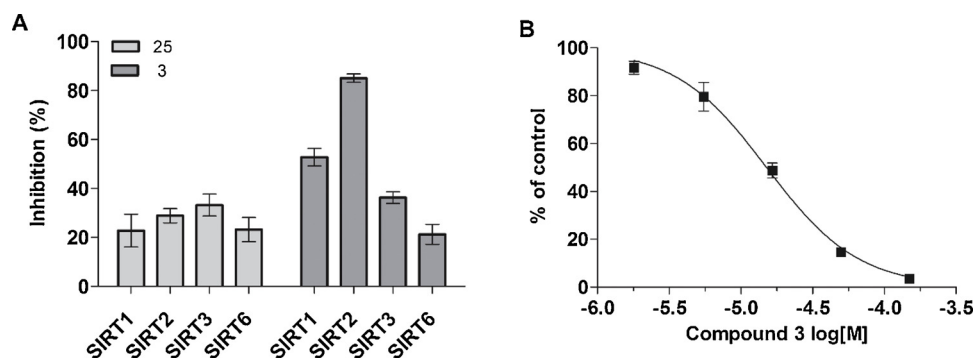


**Fig. 2.** Quercetin and its derivatives decreased the deacetylation activity of SIRT6 at 50 and 200  $\mu\text{M}$  concentrations. The acetylation status was evaluated by determining the remaining levels of histone H3 acetylated on lysine 9 and normalized to total H3 histone. (A) Images of immunoblot analysis. (B) Relative SIRT6 H3K9Ac deacetylation activity. Values are expressed as mean  $\pm$  standard error of mean (SEM) of three independent experiment (\*p values < 0.05 vs. control, \*\*p values < 0.01 vs. control; one way-ANOVA with Bonferroni and Dunnett post hoc test). (C) Structure of quercetin, compound 25 and 3.



**Fig. 3.** Inhibition of SIRT6 deacetylation activity by compounds 25 and 3. (A) Dose response curves for inhibition with different compounds concentrations. The  $\text{IC}_{50}$  values of compounds 25 and 3 were 130  $\mu\text{M}$  (113–165  $\mu\text{M}$ , 95% confidence interval) and 55  $\mu\text{M}$  (50–79  $\mu\text{M}$ , 95% confidence interval), respectively. The data is presented as mean  $\pm$  SD, (n = 3). Lineweaver-Burk plots for compounds 25 and 3 with increasing concentration of (B) peptide substrate (C)  $\text{NAD}^+$  cofactor. The data is presented as mean  $\pm$  SEM, (n = 3).





**Fig. 4.** (A) Compound **3** was more potent inhibitor towards SIRT1-3 than SIRT6. Sirtuin screen was constructed using fluorogenic based assay with recombinant enzymes at 50  $\mu$ M concentration of compounds. (B) Dose response curve for SIRT2 inhibition with different concentrations of compound **3** that provides the  $IC_{50}$  value of 14  $\mu$ M (13–16  $\mu$ M, 95% confidence interval). The data is presented as mean  $\pm$  SD, (n = 3).

### 3.2. In vitro studies for SIRT1–3

To investigate the selectivity of the most potent compounds **25** and **3** with respect to other SIRTs, fluorogenic *in vitro* assays were constructed against SIRT1–3 at a concentration of 50  $\mu$ M (Supplementary Table S2). Compound **25** displayed 20–30% inhibition against SIRT1–3 and SIRT6, whereas compound **3** showed 80% and 60% inhibition against SIRT2 and SIRT1, respectively (Fig. 4A). The dose-response analysis for compound **3** against SIRT2 showed an  $IC_{50}$  value of 14  $\mu$ M (Fig. 4B). Interestingly, compound **3** displayed 72% inhibition towards SIRT2, whereas known SIRT inhibitors Ex-527 and Suramin showed 46% and 29% inhibition, respectively, at a concentration of 10  $\mu$ M (Supplementary Table S3).

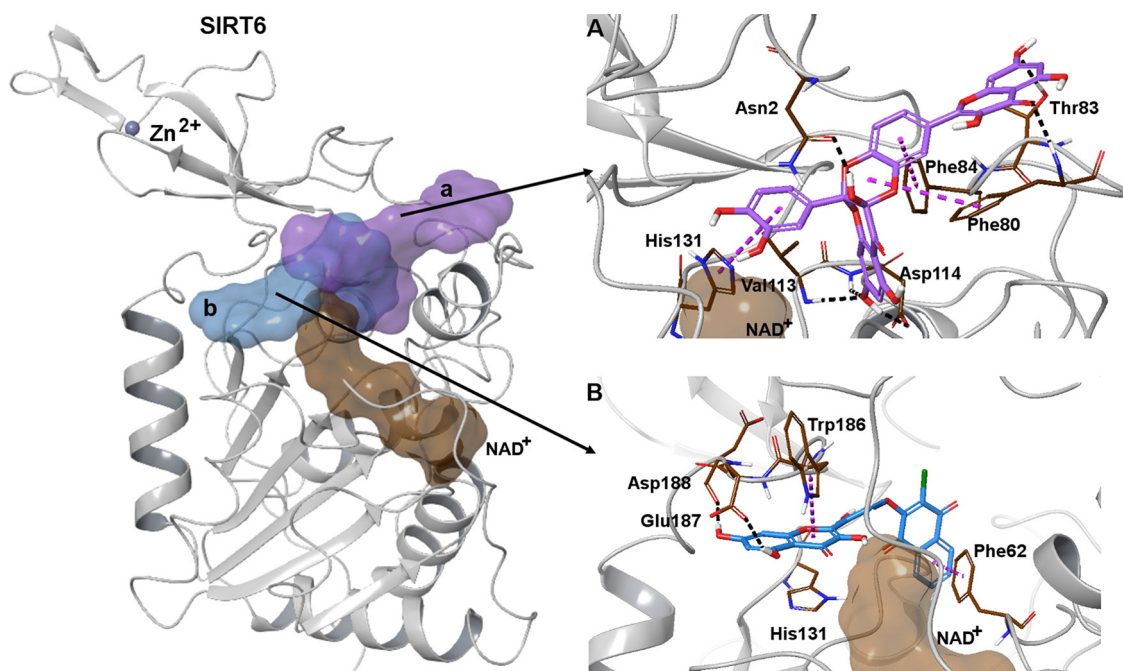
### 3.3. Molecular docking study

The most potent inhibitors, compounds **25** and **3**, were docked to SIRT6. All four isomers of compound **25** docked to the inhibitor binding site locating close to NAM moiety binding site. The isomer with best glide docking score (-12.862) formed interactions with Asn2, Phe80, Thr83, Phe84, Val113, Asp114, and His131 (Fig. 5A). His131 is the catalytically active histidine, that interacts with cofactor  $NAD^+$  during deacetylation reaction. Thus, by interacting with His131 compound **25**

might disturb the reaction between  $NAD^+$  and His131. Compound **3** did not dock as well to this binding site; the lipophilic 2-chloro-1,4-naphthoquinone moiety directed into the solvent outside of the binding site. Two-dimensional interaction figures of these compounds at this binding site are presented in Supplementary Fig. S2.

At the substrate binding site compound **3** formed interactions with Trp186, Glu187, and Asp188, and interacted also with Phe62 (Fig. 5B). Only one isomer of compound **25** had a pose at the substrate binding site, where it formed interactions with Asp185, Glu187, and Arg218. At the same time, it formed interactions also with Asn2, Asp12, Phe62, Arg63, Val113, and Asp114. Two-dimensional interactions of these compounds at the substrate binding site are presented in Supplementary Fig. S3.

Compound **25** isomers and compound **3** were docked also to SIRT2 at the binding site of co-crystallized ligand SirReal2, but were placed slightly differently than SirReal2 did (Supplementary Fig. 4A). Some isomers of compound **25** had similar interactions as SirReal2, which had three  $\pi$ - $\pi$  staking interactions to Phe131, Phe190 and Phe234. Compound **3** did not form interactions similar to SirReal2. Two-dimensional interactions of SirReal2, compound **25**, and compound **3** are presented in Supplementary Figs. 4B–D.



**Fig. 5.** Docking sites near to the binding site of NAM moiety of  $NAD^+$  (a) and near to substrate binding site (b).  $NAD^+$  binding site is close the two docking sites. (A) Interactions of compound **25** at the binding site near to NAM moiety binding site. Black dashes indicate hydrogen bonding and magenta dashes indicate  $\pi$ - $\pi$  stacking. (B) Interactions of compound **3** at substrate binding site.

## 4. Discussion

Compared to SIRT1–3, only a few SIRT6 regulators have been described in the literature to date [17,34,35,39–41]. The lack of SIRT6 regulators retards the development of clinically relevant compounds. Thus, there is an increasing need for identifying various scaffolds to be used as starting points for developing potent and selective SIRT6 inhibitors and activators. We demonstrated in a recent study that different classes of flavonoids either inhibit or activate the deacetylation activity of SIRT6. Moreover, the effect depends on the flavonoid subclass; that is, catechins showed inhibition, whereas anthocyanidins showed activation [17]. In addition, some of the flavonones and flavonols, including kaempferol and quercetin, show a dual role against SIRT6; that is, they both inhibit and activate SIRT6 depending on the substrate concentration [17].

In the present study, the SIRT6 activity of kaempferol and quercetin was tested using two different assays with different substrates. Both assays showed that quercetin and some quercetin derivatives inhibited SIRT6. Kaempferol was a weak activator according to the immunoblotting assay but a activator according to the fluorogenic-based assay. In the fluorogenic-based assay, the substrate was an H3 peptide sequence [Ac-RYQK (Ac)-AMC] that mimics the biological deacetylation site of histone 3 lysine 56 (H3K56) with Nε-acetylated lysine. However, the substrate of the immunoblotting assay consisted of the core histone including the full-length H3 peptide. The inhibition or activation results of SIRT6 may be dependent on the substrate used.

In the deacetylation reaction, SIRT6s transfer the acetyl group from the lysine residue of the substrate to the adenosine diphosphate (ADP) ribose moiety of NAD<sup>+</sup>. This process is followed by the release of NAM, O-acetyl-ADP ribose, and deacetylated substrates. The NAM moiety of NAD<sup>+</sup> and the acetyl group of the substrate lysine are placed facing towards each other and are adjacent to catalytic histidine residue involved in the reaction. Our experiments (Fig. 3) show that compound 25 displayed a competitive behavior with NAD<sup>+</sup>, suggesting that compound 25 could prevent the binding of NAD<sup>+</sup> or NAM and subsequently prevent the reaction. On the contrary, compound 3 competed with the peptide substrate, indicating that compound 3 would bind onto the binding site of substrate.

A mechanistic explanation is not possible based on these simplified models, but the results provide information about possible compound binding sites. In addition, inhibitor binding can rarely be described with one model. However, a common model of enzyme inhibition kinetics is a mixed inhibition type that represents a combination of competitive and noncompetitive inhibition [42]. Our molecular docking studies support these findings. The docking results suggest that compound 25 preferred the binding site of NAM moiety, where it formed interaction with catalytic histidine (His131). This result may indicate that compound 25 can compete with NAD<sup>+</sup> by preventing NAD<sup>+</sup> from interacting with catalytic histidine. In contrast, compound 3 preferred to dock onto the acetylated substrate binding site and formed interactions with Trp186, Glu187 and Asp188 locating in β6/α6 loop. The importance of this loop region for substrate binding has been previously discussed [17,43,44]. Both compounds 25 and 3 were also docked onto the binding site of SirReal2 in SIRT2, but they were situated slightly differently compared with SirReal2. Compound 25 had an interaction with Phe190 similar to that of SirReal2. Additionally, compound 25 formed fewer hydrogen bonds at SIRT2 than at SIRT6.

Quercetin and related flavonoids are broad-spectrum inhibitors of many protein kinases and cyclooxygenase [45,46], but understanding the mechanism of action of quercetin requires the description of its entire target space. In previous studies, quercetin derivatives (21 compounds, including all agents studied here) were tested *in vitro* to evaluate radical scavenging activity [47]. The most potent compounds of this study, compounds 25 and 3, decreased SERCA activity, which may also contribute to anticancer activity [48–50]. The compound with the most pro-oxidant properties, compound 3, exerted an antitumor

effect that was confirmed in colorectal cancer cell lines HCT-116 and HT-29 [51].

Quercetin may be a promising compound for the management of type 2 diabetes. It has been observed that aldose reductase (ALR2) inhibitors play a significant role against diabetic complications [52]. Thus, quercetin derivatives were analyzed for their ALR2 inhibitory effects [10], where compound 3 exhibited the highest biological activity. In the studies of Milackova et al. [12] and Soltesova-Prnova et al. [13], ALR2 inhibition by compounds 3 and 2 was examined in more detail. The anti-inflammatory effects were indicated in the microglial cell line (BV-2) as well as *in vivo* for compound 2 and compound 3, respectively [53,54]. Taken together, these studies indicate that polyphenols are multi-targeting compounds that affect proteins associated with cancer and diabetes. Polyphenols may have broad therapeutic implications for cancer and other age-related diseases.

## 5. Conclusions

Polyphenols are known sirtuin modulators. Several polyphenols were screened *in vitro* to detect new modulators for SIRT6. Two quercetin derivatives, diquercetin and 2-chloro-1,4-naphthoquinone-quercetin, showed inhibition towards the deacetylation activity of SIRT6. The docking results of compounds 25 and 3 support the *in vitro* results. The most potent compounds were also tested for other SIRT6s and were found to be even more potent towards SIRT2 but not towards SIRT1.

These new compounds can be used for developing more potent SIRT6 inhibitors. Future studies are required to understand the effects of polyphenols in SIRT6-mediated mechanisms of various abnormal cellular and biological functions. Overall, co-targeting of SIRT6 with other proteins/oncogenes by dietary polyphenols opens an efficient, logical, and alternative approach against many age-related diseases.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Acknowledgements

We thank Sari Ukkonen for her skillful assistance. The computational capacity and licenses support provided by CSC-IT Center for Science, Finland, is acknowledged. The research was done in collaboration network of COST Action CM1407. M.R.R. was supported by Academy of Finland (grant no. 269341), Finnish Cultural Foundation and Maud Kuistila Memorial Foundation. V.H. was supported by the Slovak National grants VEGA2/0111/16. A.H. was supported by the < GS3 > National Research, Development and Innovation Office, Hungary < /GS4 > (NKFIH; < GN3 > K119770 < /GN4 >). Ministry of Human Capacities, Hungary grant 20391-3/2018/FEKUSTRAT is acknowledged.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2019.01.035>.

## References

- [1] Y. Kanfi, S. Naiman, G. Amir, V. Peshti, G. Zinman, L. Nahum, Z. Bar-Joseph, H.Y. Cohen, The sirtuin SIRT6 regulates lifespan in male mice, *Nature* 483 (7388) (2012) 218–221, <https://doi.org/10.1038/nature10815>.
- [2] S. Kaluski, M. Portiollo, A. Besnard, D. Stein, M. Einav, L. Zhong, U. Ueberham, T. Arendt, R. Mostoslavsky, A. Sahay, D. Toiber, Neuroprotective functions for the histone deacetylase SIRT6, *Cell Rep.* 18 (13) (2017) 3052–3062, <https://doi.org/10.1016/j.celrep.2017.03.008>.
- [3] L. Tasselli, W. Zheng, K.F. Chua, SIRT6: novel mechanisms and links to aging and disease, *Trends Endocrinol. Metab.* 28 (3) (2017) 168–185, <https://doi.org/10.1016/j.tem.2016.10.002>.
- [4] C. Sebastian, B.M.M. Zwaans, D.M. Silberman, M. Gymrek, A. Goren, L. Zhong,

- O. Ram, J. Truelove, A.R. Guimaraes, D. Toiber, C. Cosentino, J.K. Greenon, A.I. MacDonald, L. McGlynn, F. Maxwell, J. Edwards, S. Giacosa, E. Guccione, R. Weissleder, B.E. Bernstein, A. Regev, P.G. Shiels, D.B. Lombard, R. Mostoslavsky, The histone deacetylase SIRT6 is a novel tumor suppressor that controls cancer metabolism, *Cell* 151 (6) (2012) 1185–1199, <https://doi.org/10.1016/j.cell.2012.10.047>.
- [5] B. Lerrer, A.A. Gertler, H.Y. Cohen, The complex role of SIRT6 in carcinogenesis, *Carcinogenesis* 37 (2) (2016) 108–118, <https://doi.org/10.1093/carcin/bgv167>.
- [6] V. Desantis, A. Lamanuzzi, A. Vacca, The role of SIRT6 in tumors, *Haematologica* 103 (1) (2018) 1–4, <https://doi.org/10.3324/haematol.2017.182675>.
- [7] M. Imran, H. Khan, M. Shah, R. Khan, F. Khan, Chemical composition and antioxidant activity of certain *Morus* species, *J. Zhejiang Univ. Sci. B* 11 (12) (2010) 973–980, <https://doi.org/10.1631/jzus.B1000173>.
- [8] I.I. da S. Junior, H. de M. Barbosa, D.C.R. Carvalho, R. de A. Barros, F.P. Albuquerque, D.H.A. da Silva, G.R. Souza, N.A.C. Souza, L.A. Rolim, F.M.M. Silva, G.L.B.P. Duarte, J.R.G. da S. Almeida, F.M. de O. Junior, D.A. Gomes, E.C. Lira, Brazilian *Morus nigra* attenuated hyperglycemia, dyslipidemia, and prooxidant status in alloxan-induced diabetic rats, *Sci. World J.* 2017 (2017) 5275813, <https://doi.org/10.1155/2017/5275813>.
- [9] I. Turan, S. Demir, K. Kilinc, N.A. Burnaz, S.O. Yaman, K. Akbulut, A. Mentese, Y. Aliyazicioglu, O. Deger, Antiproliferative and apoptotic effect of *Morus nigra* extract on human prostate cancer cells, *Saudi Pharm. J.* 25 (2) (2017) 241–248, <https://doi.org/10.1016/j.jsps.2016.06.002>.
- [10] M. Veverka, J. Galovic, E. Svajdenka, E. Veverkova, N. Pronayova, I. Milackova, M. Stefek, Novel quercetin derivatives: synthesis and screening for anti-oxidant activity and aldose reductase inhibition, *Chem. Pap.* 67 (1) (2013) 76–83, <https://doi.org/10.2478/s11696-012-0240-5>.
- [11] P. Zizkova, M. Stefek, L. Rackova, M. Prnova, L. Horakova, Novel quercetin derivatives: from redox properties to promising treatment of oxidative stress related diseases, *Chem. Biol. Interact.* 265 (2017) 36–46, <https://doi.org/10.1016/j.cbi.2017.01.019>.
- [12] I. Milackova, M. Soltesova Prnova, M. Majekova, R. Sotnikova, M. Stasko, L. Kovackikova, S. Banerjee, M. Veverka, M. Stefek, 2-Chloro-1,4-naphthoquinone derivative of quercetin as an inhibitor of aldose reductase and anti inflammatory agent, *J. Enzyme Inhib. Med. Chem.* 30 (1) (2015) 107–113, <https://doi.org/10.3109/14756366.2014.892935>.
- [13] M. Soltesova-Prnova, I. Milackova, M. Stefek, 3'-O-(3-Chloropivaloyl)quercetin,  $\alpha$ -glucosidase inhibitor with multi-targeted therapeutic potential in relation to diabetic complications, *Chem. Pap.* 70 (11) (2016) 1439–1444, <https://doi.org/10.1515/chempap-2016-0078>.
- [14] K.T. Howitz, K.J. Bitterman, H.Y. Cohen, D.W. Lamming, S. Lavu, J.G. Wood, R.E. Zipkin, P. Chung, A. Kisielewski, L.-L. Zhang, B. Scherer, D.A. Sinclair, Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan, *Nature* 425 (6954) (2003) 191–196, <https://doi.org/10.1038/nature01960>.
- [15] N. Trevino-Saldana, G. Garcia-Rivas, Regulation of sirtuin-mediated protein deacetylation by cardioprotective, *Phytochem. Oxid. Med. Cell. Longev.* 2017 (2017) 1750306, <https://doi.org/10.1155/2017/1750306>.
- [16] A.E. Peredo-Escarcega, V. Guarnier-Lans, I. Perez-Torres, S. Ortega-Ocampo, E. Carreon-Torres, V. Castrejon-Tellez, E. Diaz-Diaz, M.E. Rubio-Ruiz, The combination of resveratrol and quercetin attenuates metabolic syndrome in rats by modifying the serum fatty acid composition and by upregulating SIRT 1 and SIRT 2 expression in white adipose tissue, *Evid. Complement. Alternat. Med.* 2015 (2015) 474032, <https://doi.org/10.1155/2015/474032>.
- [17] M. Rahnasto-Rilla, J. Tyni, M. Huovinen, E. Jarho, T. Kulikowicz, S. Ravichandran, V.A. Bohr, L. Ferrucci, M. Lahtela-Kakkonen, R. Moaddel, Natural polyphenols as sirtuin 6 modulators, *Sci. Rep.* 8 (2018) 4163, <https://doi.org/10.1038/s41598-018-22388-5>.
- [18] Y.T. Lin, J.S. Yang, H.J. Lin, T.W. Tan, N.Y. Tang, J.H. Chaing, Y.H. Chang, H.F. Lu, J.G. Chung, Baicalein induces apoptosis in SCC-4 human tongue cancer cells via a  $Ca^{2+}$ -dependent mitochondrial pathway, *In Vivo (Brooklyn)* 21 (6) (2007) 1053–1058.
- [19] O.A. Ogunbayo, R.M. Harris, R.H. Waring, C.J. Kirk, F. Michelangeli, Inhibition of the sarcoplasmic/endoplasmic reticulum  $Ca^{2+}$ -ATPase by flavonoids: a quantitative structure-activity relationship study, *IUBMB Life* 60 (12) (2008) 853–858, <https://doi.org/10.1002/iub.132>.
- [20] L. Horakova, M.K. Strosova, C.M. Spickett, D. Blaskovic, Impairment of calcium ATPases by high glucose and potential pharmacological protection, *Free Radic. Res.* 47 (Suppl. 1) (2013) 81–92, <https://doi.org/10.3109/10715762.2013.807923>.
- [21] X. Xiong, G. Wang, R. Tao, P. Wu, T. Kono, K. Li, W.-X. Ding, X. Tong, S.A. Tersey, R.A. Harris, R.G. Mirmira, C. Evans-Molina, X. Charlie Dong, Sirtuin 6 regulates glucose-stimulated insulin secretion in mouse pancreatic beta cells, *Diabetologia* 59 (1) (2016) 151–160, <https://doi.org/10.1007/s00125-015-3778-2>.
- [22] M. Danilova, M. Veverka, E. Sturdik, S. Jantova, Antioxidant action and cytotoxicity on HeLa and NIH-3T3 cells of new quercetin derivatives, *Interdiscip. Toxicol.* 6 (4) (2014) 209–216, <https://doi.org/10.2478/intox-2013-0031>.
- [23] J. Viskupicova, M. Majekova, L. Horakova, Inhibition of the sarco/endoplasmic reticulum  $Ca^{2+}$ -ATPase (SERCA1) by rutin derivatives, *J. Muscle Res. Cell. Motil.* 36 (2) (2015) 183–194, <https://doi.org/10.1007/s10974-014-9402-0>.
- [24] J. Viskupicova, M.K. Strosova, P. Zizkova, M. Majekova, L. Horakova, Rutin stimulates sarcoplasmic reticulum  $Ca^{2+}$ -ATPase activity (SERCA1) and protects SERCA1 from peroxynitrite mediated injury, *Mol. Cell. Biochem.* 402 (1–2) (2015) 51–62, <https://doi.org/10.1007/s11010-014-2313-y>.
- [25] V. Heger, J. Viskupicova, Z. Zoofishan, A. Hunyadi, L. Horakova, Sarco/endoplasmic  $Ca^{2+}$ -ATPase (SERCA) and pancreatic beta cells modified by prenylated phenolic compounds from *Morus nigra*, *Biomed. Biopharm. Res.* 14 (2) (2017) 282, <https://doi.org/10.19277/bbr.14.2.168>.
- [26] A. Raza, X. Xu, L. Xia, C. Xia, J. Tang, Z. Ouyang, Quercetin-iron complex: synthesis, characterization, antioxidant, DNA binding, DNA cleavage, and antibacterial activity studies, *J. Fluoresc.* 26 (6) (2016) 2023–2031, <https://doi.org/10.1007/s10895-016-1896-y>.
- [27] J. Viskupicova, M. Danilova, M. Ondrejovic, T. Liptaj, E. Sturdik, Lipophilic rutin derivatives for antioxidant protection of oil-based foods, *Food Chem.* 123 (1) (2010) 45–50, <https://doi.org/10.1016/j.foodchem.2010.03.125>.
- [28] J.Y. Kim, W.S. Lee, Y.S. Kim, M.J. Curtis-Long, B.W. Lee, Y.B. Ryu, K.H. Park, Isolation of cholinesterase-inhibiting flavonoids from *Morus lhou*, *J. Agric. Food Chem.* 59 (9) (2011) 4589–4596, <https://doi.org/10.1021/jf200423g>.
- [29] S.H. Jeong, Y.B. Ryu, M.J. Curtis-Long, H.W. Ryu, Y.S. Baek, J.E. Kang, W.S. Lee, K.H. Park, Tyrosinase inhibitory polyphenols from roots of *Morus lhou*, *J. Agric. Food Chem.* 57 (4) (2009) 1195–1203, <https://doi.org/10.1021/jf8033286>.
- [30] H.J. Lee, D.H. Lyu, U. Koo, S.-J. Lee, S.S. Hong, K. Kim, K.H. Kim, D. Lee, W. Mar, Inhibitory effect of 2-arylbenzofurans from the Mori Cortex Radicis (Moraceae) on oxygen glucose deprivation (OGD)-induced cell death of SH-SY5Y cells, *Arch. Pharm. Res.* 34 (8) (2011) 1373–1380, <https://doi.org/10.1007/s12272-011-0818-4>.
- [31] G.D.W.F. Kapche, C.D. Fozing, J.H. Donfack, G.W. Fotso, D. Amadou, A.N. Tchana, M. Bezaib, P.F. Moundipa, B.T. Ngadju, B.M. Abegaz, Prenylated arylbenzofuran derivatives from *Morus mesozygia* with antioxidant activity, *Phytochemistry* 70 (2) (2009) 216–221, <https://doi.org/10.1016/j.phytochem.2008.12.014>.
- [32] M. Alsgo, A. Kivrak, M. Konus, C. Yilmaz, A. Kurt-Kizildogan, Synthesis and biological evaluation of novel benzothiophene derivatives, *J. Chem. Sci. Bangalore (Bangalore)* 130 (9) (2018) 119, <https://doi.org/10.1007/s12039-018-1523-3>.
- [33] M. Alsgo, A. Kivrak, New strategy for the synthesis of 3-ethynyl-2-(thiophen-2-yl) benzo[b]thiophene derivatives, *Chem. Papers* (2018), <https://doi.org/10.1007/s11696-018-0640-2>.
- [34] P. Kokkonen, M. Rahnasto-Rilla, P. Mellini, E. Jarho, M. Lahtela-Kakkonen, T. Kokkola, Studying SIRT6 regulation using H3K56 based substrate and small molecules, *Eur. J. Pharm. Sci.* 63 (2014) 71–76, <https://doi.org/10.1016/j.ejps.2014.06.015>.
- [35] M.K. Rahnasto-Rilla, P. McLoughlin, T. Kulikowicz, M. Doyle, V.A. Bohr, M. Lahtela-Kakkonen, L. Ferrucci, M. Hayes, R. Moaddel, The Identification of a SIRT6 Activator from Brown Algae *Fucus distichus*, *Mar. Drugs* 15 (6) (2017) 190, <https://doi.org/10.3390/md15060190>.
- [36] Small-Molecule Drug Discovery Suite 2017–4, Schrödinger, LLC, New York, NY, 2017.
- [37] H. Jiang, S. Khan, Y. Wang, G. Charron, B. He, C. Sebastian, J. Du, R. Kim, E. Ge, R. Mostoslavsky, H.C. Hang, Q. Hao, H. Lin, Sirt6 regulates TNF $\alpha$  secretion via hydrolysis of long chain fatty acyl lysine, *Nature* 496 (7443) (2013) 110–113, <https://doi.org/10.1038/nature12038>.
- [38] T. Rumpf, M. Schiedel, B. Karaman, C. Roessler, B.J. North, A. Lehotzky, J. Olah, K.I. Ladwein, K. Schmidkunz, M. Gajer, M. Pannek, C. Steegborn, D.A. Sinclair, S. Gerhardt, J. Ovadi, M. Schutkowski, W. Sippl, O. Einsle, M. Jung, Selective Sirt2 inhibition by ligand-induced rearrangement of the active site, *Nat. Commun.* 6 (2015) 6263, <https://doi.org/10.1038/ncomms7263>.
- [39] M. Yasuda, D.R. Wilson, S.D. Fugmann, R. Moaddel, Synthesis and characterization of SIRT6 protein coated magnetic beads: identification of a novel inhibitor of SIRT6 deacetylase from medicinal plant extracts, *Anal. Chem.* 83 (19) (2011) 7400–7407, <https://doi.org/10.1021/ac201403y>.
- [40] P. Kokkonen, M. Rahnasto-Rilla, P.H. Kiviranta, T. Huhtiniemi, T. Laitinen, A. Poso, E. Jarho, M. Lahtela-Kakkonen, Peptides and pseudopeptides as SIRT6 deacetylation inhibitors, *ACS Med. Chem. Lett.* 3 (12) (2012) 969–974, <https://doi.org/10.1021/ml300139n>.
- [41] G. Sociali, L. Galeno, M.D. Parenti, A. Grozio, I. Bauer, M. Passalacqua, S. Boero, A. Donadini, E. Millo, M. Bellotti, L. Sturla, P. Damonte, A. Puddu, C. Ferroni, G. Varchi, C. Franceschi, A. Ballestrero, A. Poggi, S. Bruzzone, A. Nencioni, A. Del Rio, Quinazolinone SIRT6 inhibitors sensitize cancer cells to chemotherapeutics, *Eur. J. Med. Chem.* 102 (2015) 530–539, <https://doi.org/10.1016/j.ejmech.2015.08.024>.
- [42] H.I. Segel, *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, Wiley-Interscience, New York, USA, 2007.
- [43] P.W. Pan, J.L. Feldman, M.K. Devries, A. Dong, A.M. Edwards, J.M. Denu, Structure and biochemical functions of SIRT6, *J. Biol. Chem.* 286 (16) (2011) 14575–14587, <https://doi.org/10.1074/jbc.M111.218990>.
- [44] H. Yuan, R. Marmorstein, Structural basis for sirtuin activity and inhibition, *J. Biol. Chem.* 287 (51) (2012) 42428–42435, <https://doi.org/10.1074/jbc.R112.372300>.
- [45] V. Garcia-Mediavilla, I. Crespo, P.S. Collado, A. Esteller, S. Sanchez-Campos, M.J. Tunon, J. Gonzalez-Gallego, The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappaB pathway in Chang Liver cells, *Eur. J. Pharmacol.* 557 (2–3) (2007) 221–229, <https://doi.org/10.1016/j.ejphar.2006.11.014>.
- [46] G.L. Russo, M. Russo, C. Spagnuolo, I. Tedesco, S. Bilotto, R. Iannitti, R. Palumbo, Quercetin: a pleiotropic kinase inhibitor against cancer, in: V. Zappia, S. Panico, G. Russo, A. Budillon, F. Della Ragione (Eds.), *Advances in Nutrition and Cancer*, Springer, Berlin, Heidelberg, 2014, pp. 185–205, [https://doi.org/10.1007/978-3-642-38007-5\\_11](https://doi.org/10.1007/978-3-642-38007-5_11).
- [47] I. Milackova, L. Kovackikova, M. Veverka, J. Galovic, M. Stefek, Screening for antiradical efficiency of 21 semi-synthetic derivatives of quercetin in a DPPH assay, *Interdiscip. Toxicol.* 6 (1) (2013) 13–17, <https://doi.org/10.2478/intox-2013-0003>.
- [48] D. Blaskovic, F. Drzik, J. Viskupicova, M. Veverka, L. Horakova, Effects of novel quercetin derivatives on sarco/endoplasmic reticulum  $Ca^{2+}$ -ATPase activity, *Free Radic. Biol. Med.* 53 (Suppl. 1) (2012) S92–S93, <https://doi.org/10.1016/j.frbm.2012.07.001>.

- freeradbiomed.2012.08.193.
- [49] D. Blaskovic, P. Zizkova, F. Drzik, J. Viskupicova, M. Veverka, L. Horakova, Modulation of rabbit muscle sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase activity by novel quercetin derivatives, *Interdiscip. Toxicol.* 6 (1) (2013) 3–8, <https://doi.org/10.2478/intox-2013-0001>.
- [50] P. Zizkova, D. Blaskovic, M. Majekova, L. Svorc, L. Rackova, L. Ratkovska, M. Veverka, L. Horakova, Novel quercetin derivatives in treatment of peroxynitrite-oxidized SERCA1, *Mol. Cell. Biochem.* 386 (1–2) (2014) 1–14, <https://doi.org/10.1007/s11010-013-1839-8>.
- [51] S. Enayat, M. Seyma Ceyhan, B. Taskoparan, M. Stefek, S. Banerjee, CHNQ, a novel 2-Chloro-1,4-naphthoquinone derivative of quercetin, induces oxidative stress and autophagy both *in vitro* and *in vivo*, *Arch. Biochem. Biophys.* 596 (2016) 84–98, <https://doi.org/10.1016/j.abb.2016.03.004>.
- [52] W.H. Tang, K.A. Martin, J. Hwa, Aldose reductase, oxidative stress, and diabetic mellitus, *Front. Pharmacol.* 3 (2012) 87, <https://doi.org/10.3389/fphar.2012.00087>.
- [53] M. Kuniakova, N. Mrvova, V. Knezl, L. Rackova, Effect of novel quercetin pivaloyl ester on functions of adult rat microglia, *Biologia* 70 (5) (2015) 690–702, <https://doi.org/10.1515/biolog-2015-0082>.
- [54] N. Mrvova, M. Skandik, M. Kuniakova, L. Rackova, Modulation of BV-2 microglia functions by novel quercetin pivaloyl ester, *Neurochem. Int.* 90 (2015) 246–254, <https://doi.org/10.1016/j.neuint.2015.09.005>.