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N-Acylethanolamines bind to SIRT6

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Abstract

Sirtuin 6 (SIRT6) is an NAD⁺-dependent histone deacetylase enzyme that is involved in multiple molecular pathways related to aging. Initially, it was reported that SIRT6 selectively deacetylated H3K9Ac and H3K56Ac, however, more recently it has been shown to preferentially hydrolyze long-chain fatty acyl groups over acetyl groups *in vitro*. Subsequently, fatty acids were demonstrated to increase the catalytic activity of SIRT6. In this study we investigated whether a series of N-acylethanolamines (NAEs), quercetin and luteolin could regulate SIRT6 activity. NAEs increased SIRT6 activity, with oleoylethanolamide having the strongest activity with an EC₅₀ of 3.1 μM. Quercetin and luteolin were demonstrated to have dual functions with respect to SIRT6 activity, namely they inhibited SIRT6 activity with an IC₅₀ of 24 μM and 2 μM, respectively, and stimulated SIRT6 activity (over 6 fold) with an EC₅₀ of 990 μM and 270 μM, respectively.

Keywords

enzymes; ethanolamides; natural product; quercetin; SIRT6 activators

Introduction

Sirtuins are NAD⁺-dependent histone deacetylase enzymes (HDACs) that function as regulators of many cellular processes [1]. To date, seven sirtuins (SIRT1-7) have been identified [1a], that are evolutionarily conserved, existing in a variety of organisms from eukaryote to humans. The catalytic activity of sirtuins has been closely associated with deacetylation of histones, however, they have also been shown to deacetylate nonhistone proteins [2], demonstrating their role in pathways controlling aging, cancers and metabolic diseases [1a].

Although SIRT6 has been less studied than SIRT1-3, it has gained interest in age-associated diseases because of its role in various cellular functions, such as genomic stability, oxidative

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stress and glucose metabolism [3]. SIRT6-deficient mice presented with a premature aging phenotype accompanied with a shortened lifespan [1b, 4], while overexpression of SIRT6 prolonged the lifespan of male mice and prevented physiological damage caused by diet induced obesity [5]. SIRT6 was shown to play a role in several cancer subtypes either, as a tumor suppressor or promoter, or both [6]. Consequently SIRT6 has been identified as a potential therapeutic target in the context of age-associated diseases, cancer and metabolic disorders including obesity and insulin resistant diabetes [5, 7].

In addition to its deacetylation activity, SIRT6 possesses mono-ADP-ribosyltransferase activity [8] and, carries out deacylation of long-chain fatty acyl groups from lysines [9]. In fact, the catalytic activity of SIRT6 was demonstrated to preferentially hydrolyze long-chain fatty acyl groups over deacetylation *in vitro* [9], by a factor of 300. Further, it was found that while deacetylation was specific to H3K9 and H3K56, hydrolysis of the corresponding myristoyl peptide was observed for the aforementioned peptides as well as the H2BK12 sequence [9]. Subsequently, Denu and collaborators found that fatty acids: oleic, myristic, palmitic, stearic, linoleic and α -linolenic acids increased SIRT6 deacetylation activity [10], with myristic acid increasing deacetylase activity by the greatest magnitude (10 fold) with an EC₅₀ of 246 μ M, and oleic and linoleic acids being the most effective at increasing SIRT6 activity with an EC₅₀= ~100 μ M, albeit by only 5-6 fold.

SIRT6 has also been shown to be involved in rosiglitazone mediated metabolic regulation [11]. When rosiglitazone, a peroxisome proliferator-activated receptor (PPAR)- γ agonist, was administered to rats, it increased the expression of SIRT6, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) and forkhead box O1 (Foxo1) protein as well as causing increases in both liver kinase B1 (LKB1) and 5'adenosine monophosphate-activated protein kinase (AMPK) activities. When SIRT6 was absent the effects of rosiglitazone on hepatocyte fat accumulation ceased, suggesting rosiglitazone protects against hepatic steatosis by activating the SIRT6-AMPK pathway. The data suggested interactions between the nuclear receptor PPAR- γ and SIRT6. Similarly, Jin et al, demonstrated that fisetin, a flavanol, increased deacetylation of PGC-1 α via SIRT1, and as a result increased its interaction with the PPAR complex and increased transcription [12]. Several other flavonoids, including quercetin and luteolin, were also identified as SIRT6 inhibitors [13].

The biological activity of endogenous fatty acid ethanolamides, N-acylethanolamines (NAEs), has recently been demonstrated to be mediated by PPAR- α [16-18a] and PPAR- γ [17] as well as through other targets. For example, anandamide bound to and induced PPAR- γ gene expression [14], while oleoylethanolamide bound to and increased the transcriptional activity of PPAR- α [15]. NAEs are a group of endogenous compounds involved in a variety of physiological processes, including energy homeostasis and inflammation [16].

As a result of their physiological actions and due to previous studies demonstrating that fatty acids can activate SIRT6 [9-10], a series of NAEs (Table 1), were tested for activity at the SIRT6 protein using an *in vitro* H3K9 deacetylation assay. Quercetin and luteolin, previously identified SIRT6 inhibitors were also tested for SIRT6 activity.

Results and Discussion

A SIRT6 *in vitro* assay was developed to determine the functional activity of SIRT6 regulators. The reaction conditions for the developed assay had to be optimized depending on whether inhibition or activation assays were being conducted. H3K9Ac, a H3 based substrate for SIRT6, frequently used to measure SIRT6 activity was used in this study. The ratio of the deacetylated histone (H3K9) with H3K9Ac was used to measure the activity of the enzyme. Various concentrations of H3K9Ac (10-300 μ M), SIRT6 enzyme (1-10 μ g) (Suppl Fig 1A) and incubation times were studied (Suppl Fig 1B). All the tested concentrations of SIRT6 were in a linear range, with the exception of 1 μ g/ml, which resulted in no detectable deacetylated histone. The deacetylation activity of SIRT6 (3 μ g/ml) was subsequently demonstrated to be linear from 15 min to 2 h. The initial rate velocities at varying concentrations of substrate H3K9Ac were measured and the data were fitted to the Michaelis–Menten equation to yield K_m and V_{max} values of 232 μ M and 87 pmol/mg protein/min for the H3K9Ac substrate, respectively (Figure 1).

The SIRT6 deacetylation assay was carried out with 40 μ M H3K9Ac and a 30 min incubation time when testing for activators. The assay was validated by determining the deacetylation activity in the presence of 100 μ M of a set of known SIRT6 activators, including oleic acid, linoleic acid and stearic acid (Figure 2A)^[10]. Oleic and linoleic acid were strong activators of SIRT6, while stearic acid displayed very little activity, consistent with literature^[10]. As a result, the functional activity was determined for both oleic and linoleic acid. They stimulated SIRT6 in a dose dependent manner with an EC_{50} of 89 $\pm$ 15 μ M and 230 $\pm$ 10 μ M, respectively (Figure 2B), similar to previously reported values of 90 μ M and 100 μ M^[10].

The SIRT6 inhibition assay was optimized and it was determined that 150 μ M H3K9Ac and 60 min incubation time were optimal. We have previously demonstrated that a series of flavanoids had significant inhibitory effects on the deacetylation activity of SIRT6^[13], as a result, quercetin and luteolin were used as positive controls. Both quercetin and luteolin inhibited SIRT6 deacetylase activity in a concentration-dependent manner (Table 1), with an IC_{50} value of 24 μ M and 4.1 μ M, respectively, consistent with previous reported values^[13a, 17]. Further, they both inhibited SIRT6 deacetylation activity to a similar extent (38 and 30%, respectively).

A series of ethanolamides (Scheme 1), arachidonoyl (AEA), linoleoyl (LEA), α -linolenoyl (A-LEA), γ -linolenoyl (G-LEA), myristoyl (MEA), oleoyl (OEA), palmitoleoyl (POEA), palmitoyl (PEA), stearoyl (STEA), were tested for SIRT6 activity at 100 μ M, as this concentration was effective at activating SIRT6 with the fatty acids. All the NAE's increased SIRT6 activity, with the exception of STEA and AEA, with a ~1.5 fold increase for γ -LEA to ~2.1 fold increase for OEA (data not shown). Therefore, the concentration was decreased to 10 μ M to determine which NAE's were most active at SIRT6 (Figure 3A). As can be seen OEA, MEA and PEA had increased SIRT6 activity by the greatest extent, with a 2 fold increase, similar to what was observed with 100 μ M linoleic acid (Figure 2A). Interestingly, linoleic acid was able to stimulate SIRT6 more efficiently as compared with the corresponding ethanolamide LEA. Although NAEs stimulated SIRT6 deacetylase activity at

concentrations of 10 μM , the magnitude of stimulation was more efficient with fatty acids. Similar results were obtained using the SIRT6-His tag (Suppl Fig 2), confirming that the activation was not the result of any interaction with the GST fusion protein. As OEA and MEA stimulated SIRT6 activity by the greatest extent for both the GST and His-Tag SIRT6, a dose response curve for MEA and OEA (0.02 μM to 100 μM) was carried out. SIRT6 activity increased for both MEA and OEA in a dose-dependent manner with an $\text{EC}_{50}=7.5 \pm 3 \mu\text{M}$ and $3.1 \pm 2 \mu\text{M}$, respectively (Figure 3B), with a 2 fold increase in activity (Table 1). The EC_{50} values obtained were below the circulating physiological concentrations^[18] of OEA and MEA, however, the resulting intranuclear concentration of OEA was sufficient for activation of PPAR α , where OEA has been reported to have an EC_{50} in the 100 nM to μM range and therefore could be expected to be sufficient to activate SIRT6^[19].

The dose response curve carried out to determine the IC_{50} s of quercetin and luteolin, resulted in an increase in activity at higher concentrations (>300 μM). As a result, the deacetylation activation assay was carried out for quercetin and luteolin at a concentration range from 50 μM to 10 mM. Quercetin increased the activity of SIRT6 10 fold with an EC_{50} value of 990 μM , while luteolin increased SIRT6 activity ~6 fold with an EC_{50} of 270 μM (Table 1; Figure 4).

While the EC_{50} is at a concentration is not physiologically relevant, the result indicates that quercetin may have two binding sites on SIRT6. The conserved catalytic domain of sirtuins, including SIRT6, consists of a large Rossmann fold domain and a smaller zinc-binding domain, connected by four linking loops. It has been previously demonstrated that NAD⁺ binding induces a conformational change from a catalytically inactive open conformation to an active closed conformation^[20]. The binding of the myristoylated peptide to the SIRT6 co-crystal structure has been carried out and revealed a large hydrophobic pocket that responsible for binding the long-chain fatty acyl groups^[9] to the protein. The binding of fatty acids to this pocket was shown to induce a conformational change that stimulated deacetylation activity^[10] and may also be the site where the NAEs bind. In the case of quercetin and luteolin, they may bind to SIRT6 at low concentrations preventing NAD⁺ from binding and thereby decrease SIRT6 activity, while at higher concentrations they may induce a similar conformational change induced by fatty acids that result in increased activity.

Conclusions

The identification of NAEs as SIRT6 activators, specifically, OEA and MEA with EC_{50} s of 3 μM and 4 μM , respectively, could identify a novel mechanism of action for the NAEs. Interestingly, while quercetin and luteolin both elicited a dose-dependent inhibition of SIRT6 deacetylation activity as previously reported^[17], they also displayed significant increase (6-10 fold) of SIRT6 deacetylation activity at higher doses. The identification of NAEs and the flavonoids as activators of SIRT6 expands the multiple roles of SIRT6.

Experimental

Chemicals

Acetylated histone H3 (K9) peptide (residues 1-21) (H3K9Ac) was purchased from AnaSpec Incorporation (Fremont, USA). Luteolin, nicotinamide adenine dinucleotide (NAD), formic acid, long-chain fatty acid derivatives (linoleic, oleic, and stearic) acid were ordered from Sigma Aldrich (USA). Ethanolamide derivatives (arachidonoyl, linoleoyl, α -linolenoyl, γ -linolenoyl, myristoyl, oleoyl, palmitoleoyl, palmitoyl, stearoyl) were from Cayman Chemical Company (Michigan, USA). His-tagged human recombinant SIRT6 was ordered from Biovision Inc. (Milpitas, USA).

Expression and Purification of GST-Tagged SIRT6 Protein

The human SIRT6 expression vector hSIRT6-pGEX-6P3 was kindly provided by Prof. Katrin Chua (Stanford, USA). The recombinant GST-tagged SIRT6 was produced by fermentation in *Escherichia coli* BL21(DE3)-pRARE. The production was done at +16°C with 0.1 mM IPTG for 20 h and the soluble overexpressed protein was affinity purified on glutathione agarose (Sigma).

Optimization of the SIRT6 Deacetylation Assay

To ensure optimal experimental condition, the reaction linearity at varying SIRT6 enzyme amount, reaction time or H3K9Ac substrate were determined for the assay. The reaction mixture contained 75-150 μ M H3K9Ac, 500 μ M NAD and recombinant SIRT6 (1-10 μ g/well) in Tris Buffer [25 mM, pH 8.0]. The reactions were initiated by addition of the recombinant SIRT6 to wells, incubated from 15 min to 4 hours at 37°C and quenched by adding 6 μ l of formic acid (final concentration 1%). The mixtures were centrifuged at 16 100 g for 15 min.

The linearity of reaction velocity was measured by varying H3K9Ac substrate (10-300 μ M) at various times points. Time points were selected so that steady-state initial velocities were maintained in all reactions. In the experiment H3K9Ac was incubated with SIRT6 (3 μ g/well), saturating NAD (3 mM) in Tris Buffer [25 mM, pH 8.0] at 37°C, and subsequently quenched by adding 6 μ l of formic acid (final concentration 1%). The data were fitted to Michaelis-Menten equation using Graph Pad Prism Software version 6 (California, USA). All experiments were carried out using 1 % DMSO concentration. The blank controls were carried out without recombinant SIRT6 enzyme or NAD.

HPLC Analysis

The chromatographic separation of H3K9 and acetylated H3K9 was achieved on a Zorbax Eclipse XDB-C18 column (4.6 mm $\times$ 50 mm, 1.8 μ m; Agilent Technologies, Santa Clara, CA, USA) at room temperature using a Shimadzu prominence system (Shimadzu Technology, Japan) consisting of a CBM-20A, LC-20 AB binary pumps, an SIL-20AC-HT autosampler and a DGU-20A3 degassing unit. The mobile phase consisted of water with 0.02% formic acid (elute A) and acetonitrile with 0.02% formic acid (elute B). The gradient eluent at a flow rate of 0.9 ml/min was programmed as follows: 0-2.0 min, 0% B; 2.0-10 min, 0-8% B; 10-10.10 min, 8-80% B; 10.10-12 min, 80%; 12-12.1 min 80-0% B; 15 min,

0% B. The total run time was 15 min and the injection volume per sample was 20 μ l. The HPLC system was coupled to a 5500 QTRAP from Applied Biosystems/MDS Sciex equipped with Turbo V electrospray ionization source (TIS)[®] (Applied Biosystems, Foster City, CA, USA). The data was acquired and analyzed using Analyst version 1.5.1 (Applied Biosystems). Positive electrospray ionization data were acquired using multiple reaction monitoring (MRM). The TIS instrumental source settings for temperature, curtain gas, ion source gas 1 (nebulizer), ion source gas 2 (turbo ion spray), entrance potential and ion spray voltage were 550°C, 20 psi, 60 psi, 50 psi, 10V and 5500 V, respectively. The TIS compound parameter settings for declustering potential, collision energy, and collision cell exit potential were 231 V, 45 V, and 12 V, respectively, for H3K9Ac and 36 V, 43 V and 12 V, respectively for H3K9. The standards were characterized using the following MRM ion transitions: H3K9Ac (m/z 766.339→760.690) and H3K9 (m/z 752.198→746.717).

SIRT6 Deacetylation Assay

2.5.1 Activation assay—Ligands were incubated for 30 min in the presence of 3 μ g/well of SIRT6, 40 μ M H3K9Ac and 500 μ M NAD in Tris Buffer [25 mM, pH 8.0] at 37°C. During the reaction the final solvent concentration of all samples was 1% DMSO. 100 M fatty acids (linoleic, oleic and stearic) and 10 μ M ethanolamides, (AEA), (LEA), (A-LEA), (G-LEA), (MEA), (OEA), (POEA), (PEA), (STEA), were tested against SIRT6.

EC50's were determined for linoleic acid, oleic acid, MEA, OEA and quercetin and luteolin. The concentrations of substance range from 0.07 μ M to 100 μ M for MEA and 0.02 μ M to 100 μ M OEA and from 70 μ M to 2000 μ M for linoleic acid and oleic acid and 100 μ M to 10 mM for quercetin and 50 μ M to 5 mM for luteolin. The EC50-values were calculated using Graph Pad Prism Software version 6 (California, USA).

2.5.2 Inhibition Assay—Under the defined assay condition deacetylation activity of SIRT6 were determined in the presence of quercetin. 3 μ g/well of SIRT6, 150 μ M H3K9Ac, 500 μ M NAD in Tris Buffer [25 mM, pH 8.0], were incubated for 60 min at 37°C. Concentrations were varied from 2 μ M to 250 μ M for quercetin and 0.3 μ M to 25 μ M for luteolin. The IC₅₀-values were calculated using Graph Pad Prism Software version 6 (California, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. a Haigis MC, Sinclair DA. Annu Rev Pathol. 2010; 5:253–295. [PubMed: 20078221] b Mostoslavsky R, Chua KF, Lombard DB, Pang WW, Fischer MR, Gellon L, Liu P, Mostoslavsky G, Franco S, Murphy MM, Mills KD, Patel P, Hsu JT, Hong AL, Ford E, Cheng HL, Kennedy C,

- Nunez N, Bronson R, Frendewey D, Auerbach W, Valenzuela D, Karow M, Hottiger MO, Hursting S, Barrett JC, Guarente L, Mulligan R, Demple B, Yancopoulos GD, Alt FW. *Cell*. 2006; 124:315–329. [PubMed: 16439206]
2. Cheng HL, Mostoslavsky R, Saito S, Manis JP, Gu Y, Patel P, Bronson R, Appella E, Alt FW, Chua KF. *Proc Natl Acad Sci U S A*. 2003; 100:10794–10799. [PubMed: 12960381]
3. Gertler AA, Cohen HY. *Biogerontology*. 2013; 14:629–639. [PubMed: 24213807]
4. Kim HS, Xiao C, Wang RH, Lahusen T, Xu X, Vassilopoulos A, Vazquez-Ortiz G, Jeong WI, Park O, Ki SH, Gao B, Deng CX. *Cell Metab*. 2010; 12:224–236. [PubMed: 20816089]
5. Kanfi Y, Naiman S, Amir G, Peshti V, Zinman G, Nahum L, Bar-Joseph Z, Cohen HY. *Nature*. 2012; 483:218–221. [PubMed: 22367546]
6. a Liu Y, Xie QR, Wang B, Shao J, Zhang T, Liu T, Huang G, Xia W. *Protein Cell*. 2013b Zhang ZG, Qin CY. *Mol Med Rep*. 2014; 9:882–888. [PubMed: 24366394]
7. a Rodgers JT, Puigserver P. *Cell Metab*. 2006; 3:77–78. [PubMed: 16459306] b Zhong L, D'Urso A, Toiber D, Sebastian C, Henry RE, Vadysirisack DD, Guimaraes A, Marinelli B, Wikstrom JD, Nir T, Clish CB, Vaitheesvaran B, Iliopoulos O, Kurland I, Dor Y, Weissleder R, Shirihai OS, Ellisen LW, Espinosa JM, Mostoslavsky R. *Cell*. 2010; 140:280–293. [PubMed: 20141841] c Dominy JE Jr, Lee Y, Jedrychowski MP, Chim H, Jurczak MJ, Camporez JP, Ruan HB, Feldman J, Pierce K, Mostoslavsky R, Denu JM, Clish CB, Yang X, Shulman GI, Gygi SP, Puigserver P. *Mol Cell*. 2012; 48:900–913. [PubMed: 23142079]
8. Borra MT, O'Neill FJ, Jackson MD, Marshall B, Verdin E, Foltz KR, Denu JM. *J Biol Chem*. 2002; 277:12632–12641. [PubMed: 11812793]
9. Jiang H, Khan S, Wang Y, Charron G, He B, Sebastian C, Du J, Kim R, Ge E, Mostoslavsky R, Hang HC, Hao Q, Lin H. *Nature*. 2013; 496:110–113. [PubMed: 23552949]
10. Feldman JL, Baeza J, Denu JM. *J Biol Chem*. 2013; 288:31350–31356. [PubMed: 24052263]
11. Yang SJ, Choi JM, Chang E, Park SW, Park CY. *PLoS One*. 2014; 9:e105456. [PubMed: 25133775]
12. Jin T, Kim OY, Shin MJ, Choi EY, Lee SS, Han YS, Chung JH. *J Agric Food Chem*. 2014; 62:10468–10474. [PubMed: 25286082]
13. a Yasuda M, Wilson DR, Fugmann SD, Moaddel R. *Anal Chem*. 2011; 83:7400–7407. [PubMed: 21854049] b Ravichandran S, Singh N, Donnelly D, Migliore M, Johnson P, Fishwick C, Luke BT, Martin B, Maudsley S, Fugmann SD, Moaddel R. *J Mol Graph Model*. 2014; 49:38–46. [PubMed: 24491483]
14. a Bouaboula M, Hilairat S, Marchand J, Fajas L, Le Fur G, Casellas P. *Eur J Pharmacol*. 2005; 517:174–181. [PubMed: 15987634] b Karaliota S, Siafaka-Kapadai A, Gontinou C, Psarra K, Mavri-Vavayanni M. *Obesity (Silver Spring)*. 2009; 17:1830–1838. [PubMed: 19543211]
15. O'Sullivan SE. *Br J Pharmacol*. 2007; 152:576–582. [PubMed: 17704824]
16. Balvers MG, Verhoeckx KC, Meijerink J, Wortelboer HM, Witkamp RF. *CNS Neurol Disord Drug Targets*. 2013; 12:23–33. [PubMed: 23394528]
17. Singh N, Ravichandran S, Norton DD, Fugmann SD, Moaddel R. *Anal Biochem*. 2013; 436:78–83. [PubMed: 23376017]
18. Psychogios N, Hau DD, Peng J, Guo AC, Mandal R, Bouatra S, Sinelnikov I, Krishnamurthy R, Eisner R, Gautam B, Young N, Xia J, Knox C, Dong E, Huang P, Hollander Z, Pedersen TL, Smith SR, Bamforth F, Greiner R, McManus B, Newman JW, Goodfriend T, Wishart DS. *PLoS One*. 2011; 6:e16957. [PubMed: 21359215]
19. Hansen HS, Diep TA. *Biochem Pharmacol*. 2009; 78:553–560. [PubMed: 19413995]
20. a Davenport AM, Huber FM, Hoelz A. *J Mol Biol*. 2014; 426:526–541. [PubMed: 24120939] b Yuan H, Marmorstein R. *J Biol Chem*. 2012; 287:42428–42435. [PubMed: 23086949]

Figure 1. Michaelis–Menten steady-state kinetic analyses of SIRT6 deacetylation of 10-300 μ M H3K9Ac with 3 mM NAD with 15 min (■) and 30 (●)min incubation times. The data is presented as mean $\pm$ SD, $n = 2$.

Figure 2 A-B.

SIRT6 deacetylation activity in the presence of fatty acids. **A.** Change of SIRT6 deacetylation in the presence of 100 μ M fatty acids (linoleic, oleic, stearic) with 500 μ M NAD and 40 μ M H3K9Ac with 30 min incubation time. The data is presented as mean $\pm$ SD, n = 2. **B.** Dose response curve of oleic acid (●) and linoleic acid (■) (70-2000 μ M) on SIRT6 deacetylation activity. The data is presented as mean $\pm$ SD, n = 3.

Figure 3 A-B.

SIRT6 deacetylation activity in the presence of NAEs. **A.** Change of SIRT6 deacetylation in the presence of 10 μM NAEs with 500 μM NAD and 40 μM H3K9Ac with 30 min incubation time. The data is presented as mean $\pm$ SD, $n = 2$. **B.** Dose response curve of MEA (●)(0.07 μM to 100 μM) and OEA (▲) (0.02 μM to 100 μM) on SIRT6 deacetylation activity. The data is presented as mean $\pm$ SD, $n = 3$.

Figure 4. Dose response curve of luteolin (●) (50 μ M to 5 mM) and quercetin (■) (100 μ M to 10 mM) SIRT6 deacetylation activity. The data is presented as mean $\pm$ SD, n = 3.

Scheme 1.
Structures of N-acylethanolamines (NAEs)

Table 1

The functional activity of fatty acids (linoleic acid (LA) and oleic acid (OA)), N-acylethanolamines (oleoylethanolamide (OEA), myristoylethanolamide (MEA)) and flavonoids (quercetin, luteolin) against the SIRT6 protein, with maximal stimulation reported for SIRT6 activators and maximum inhibition reported for SIRT6 inhibitors. The data is presented as mean $\pm$ SD, n = 2.

	EC ₅₀ -value $\pm$ SD (μ M)	IC ₅₀ -value $\pm$ SD (μ M)	Maximum stimulation (fold)	Maximum Inhibition (%)
LA	89 $\pm$ 15	>100 ^[a]	3.7	ND ^[b]
OA	230 $\pm$ 10	>100 ^[a]	4.6	ND ^[b]
OEA	3.1 $\pm$ 2	>100 ^[a]	2.1	ND ^[b]
MEA	7.5 $\pm$ 3	>100 ^[a]	2	ND ^[b]
Quercetin	990 $\pm$ 250	24 $\pm$ 8	10	38
Luteolin	270 $\pm$ 25	1.9 $\pm$ 4	6.1	30

^[a]No inhibition at 100 μ M concentration

^[b]Not determined