J Pharmacol Sci **108**, 364 – 371 (2008)

Journal of Pharmacological Sciences

Full Paper

A Novel Chalcone Polyphenol Inhibits the Deacetylase Activity of SIRT1 and Cell Growth in HEK293T Cells

Tomoaki Kahyo[1,*], Shuji Ichikawa[2], Takahiro Hatanaka[1], Maki K. Yamada[1], and Mitsutoshi Setou[1,3]

[1]*Mitsubishi Kagaku Institute of Life Sciences (MITILS), 11 Minamiooya, Machida, Tokyo 194-8511, Japan*
[2]*Mitsubishi Chemical Group Science and Technology Research Center, INC., 1000 Kamoshida-cho, Aoba-ku, Yokohama, Kanagawa 227-8502, Japan*
[3]*Hamamatsu University School of Medicine, Department of Molecular Anatomy, 1-20-1, Handayama, Hamamatsu, Shizuoka 431-3192, Japan*

Received August 4, 2008; Accepted September 29, 2008

Abstract. SIRT1 is one of seven mammalian orthologs of yeast silent information regulator 2 (Sir2), and it functions as a nicotinamide adenine dinucleotide (NAD)-dependent deacetylase. Recently, resveratrol and its analogues, which are polyphenols, have been reported to activate the deacetylase activity of SIRT1 in an in vitro assay and to expand the life-span of several species through Sir2 and the orthologs. To find activators or inhibitors to SIRT1, we examined thirty-six polyphenols, including stilbenes, chalcones, flavanones, and flavonols, with the SIRT1 deacetylase activity assay using the acetylated peptide of p53 as a substrate. The results showed that 3,2',3',4'-tetrahydroxychalcone, a newly synthesized compound, inhibited the SIRT1-mediated deacetylation of a p53 acetylated peptide and recombinant protein in vitro. In addition, this agent induced the hyperacetylation of endogenous p53, increased the endogenous $p21^{CIP1/WAF1}$ in intact cells, and suppressed the cell growth. These results indicated that 3,2',3',4'-tetrahydroxychalcone had a stronger inhibitory effect on the SIRT1-pathway than sirtinol, a known SIRT1-inhibitor. Our results mean that 3,2',3',4'-tetrahydroxychalcone is a novel inhibitor of SIRT1 and produces physiological effects on organisms probably through inhibiting the deacetylation by SIRT1.

Keywords: SIRT1, deacetylation, inhibitor, polyphenol, p53

Introduction

Sir2 and its orthologs (sirtuins) have been shown to expand the life span of several species (1 – 3). In *Caenorhabditis elegans*, for example, life span is extended by the presence of extra copies of a *sir*-2.1 gene (4). SIRT1, the closest mammalian homolog of yeast Sir2, is important for various cellular functions, including apoptosis, differentiation, proliferation, metabolism, and protein degradation (5 – 8). Molecularly, SIRT1 is known as a NAD-dependent histone deacetylase (HDACs) (9). Histone deacetylases are classified into three groups: the Rpd3-related class I, Hda2-related class II, and Sir2 family (class III); and NAD is necessary only for deacetylation by the Sir2 family (10, 11). In addition to histone, tumor suppressor p53, forkhead transcription factor (FOXO), and PPAR-Gamma co-activator (PGC)-1alpha are also targets for deacetylation by SIRT1 (12 – 16). As regards to p53, the acetylation has been reported to promote site-specific DNA-binding of p53, to prevent p53 from the ubiquitination by Mdm2, or to alter the localization of p53 in the nucleus (17). Therefore, the p53 deacetylation by SIRT1 affects cellular viability because p53 is a transcriptional factor acting in response to various stresses and induces cell cycle arrest or apoptosis (18, 19).

Previous studies have attempted to identify small molecular activators of sirtuins. According to a comprehensive survey using a high-throughput screen, resveratrol and its analogues, known as polyphenols, have been reported to promote the deacetylase activity of SIRT1, although the detailed mechanism of this activation remains unknown (1). In addition, it has been reported that activators of sirtuins expand the life spans of yeasts,

*Corresponding author. aynoken@mitils.jp
Published online in J-STAGE on November 13, 2008 (in advance)
doi: 10.1254/jphs.08203FP

worms, and flies (1, 3).

Inhibitors of sirtuins have also been studied. Nicotinamide is known as a product of the Sir2 deacetylation reaction and also acts as a physiological inhibitor of Sir2 (20 – 22). Sirtinol, splitomicin, some kinds of indoles and their derivatives have been identified as inhibitors of sirtuins by using a phenotypic screen in yeast and an in vitro deacetylation assay (23 – 28). In addition, some compounds were characterized as inhibitors of sirtuins by applying molecular modeling and virtual screening (29, 30). Due to their deacetylase inhibiting properties, the small molecules described above have been suggested to be useful for analysis of the catalytic mechanism of sirtuins and could be antitumor drug candidates (31, 32).

Here, we report that 3,2',3',4'-tetrahydroxychalcone (compound 17), a chalcone polyphenol here synthesized for the first time, inhibited the deacetylase activity of SIRT1 in in vitro assays, induced hyperacetylation of p53 in intact cells, and suppressed the cell growth. This result will help us to develop hydroxychalcones for anticancer drugs.

Materials and Methods

Chemical compounds

All compounds excluding 17 and 18 were commercially purchased: compounds 13, 21, 22, 23, 24, 25, and 31 were from Tokyo Chemical Industry (Tokyo); compounds 1, 2, 11, 26, 29, and 30 were from Sigma (St. Louis, MO, USA); compounds 4, 5, 6, 8, 9, and 10 were from Apin Chemicals (Oxon, UK); compounds 14, 19, 32, and 33 were from Aldrich (St. Louis, MO, USA); compounds 20, 27, and 28 were from Fluka (Buchs, Switzerland); compounds 15 and 16 were from Across (Saitama); compounds 3 and 12 were from Kanto Chemical (Tokyo); compounds 34 and 35 were from Nacalai Tesque (Kyoto); and compounds 7 and 36 were from Indofine (Hillsborough, NJ, USA) and Key Organics (Cornwall, UK), respectively.

Synthesis of compounds 17 and 18

All reactions were carried out with stirring under an atmosphere of nitrogen. The first two reactions were stopped by adding water and sat. NaHCO3 aq., and then the dried samples were treated with charcoal and evaporated in vacuo. For 2',3',4'-ris(methoxymethoxy) acetophenone (I, 13.3 g, 44.3 mmol: 89% yield), 2',3',4'-trihydroxyacetophenone (8.41 g, 50 mmol) and hexane-washed NaH (8.68 g, 217 mmol) were reacted for 1 h in tetrahydrofuran (350 mL); and then chloromethyl methyl ether (17.35 g, 216.9 mmol) was added dropwise and reacted at r.t. for further 6 h. For 2'-methoxymethoxybenzaldehyde (II, 11.3 g, 99% purity), *m*-hydroxybenzaldehyde (7.33 g, 60 mmol) and triethyl amine (9.12 g, 90 mmol) were mixed at 0°C in *N*,*N*-dimethylformamide (350 mL), and then chloromethyl methyl ether (6.39 g, 79 mmol) was added reacted at r.t. for 3 h; and then hexane-washed NaH (2.01 g, 50.3 mmol as 60% NaH: before washing) and chloromethyl methyl ether (4.14 g, 51.4 mmol) were added and further reacted for 2.5 h at 0°C. For (*E*)-1-(2-methoxymethoxyphenyl)-3-(2',3',4'-trimethoxymethoxy phenyl)-2-propene-1-one (III, 4.2 g, 9.4 mmol), the above yielded compound I (3.01 g, 10 mmol) and compound II (2.0 g, 11.9 mmol) and potassium hydroxide (14.04 g, 50.1 mmol) were dissolved in ethanol (15 mL) and reacted for 12 h at r.t. The resultant compound III (0.47 g, 1.04 mmol) was dissolved in methanol (42 mL), added with 10% HCl – methanol (3.80 g, 10.4 mmol), refluxed for 8 min, and then cooled in an ice bath. The concentrated ethyl acetate extract was applied to a silica gel column, and then compound 17 (33 mg, 0.12 mmol, yield 12%, >99% purity) or (*E*)-1-(2,4-dihydroxyphenyl)-3-(2',5'-dihydroxyphenyl)-2-propene-1-one (compound 18, 22% yield) was eluted with hexane / ethyl acetate (65:35).

In vitro deacetylase activity assay

The SIRT1 or HDACs deacetylase activity assay kit was purchased from CycLex (Nagano) and the assay was carried out according to the manufacturer's instructions. The reaction was performed at room temperature for 30 min. The deacetylase activity was detected as a fluorescent emission at 440 nm with excitation at 340 nm. The fluorescence intensity of the compounds at 440 nm was subtracted from the values measured in the assay. The resulting value was treated as the baseline value in the absence of compound. The experiments were performed at least three times.

Cell culture, transfection, and antibodies

HEK293T (human embryonic kidney cell) cells were cultured at 37°C in air with 10% CO_2. DMEM (Dulbecco's modified Eagle's medium; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) was used for the culture. Transfection was performed as described (33). All antibodies were commercially purchased: rabbit anti-p53 Ac-K382 antibodies (Cell Signaling Technology, Beverly, MA, USA); mouse anti-p21 (BD Bioscience, San Jose, CA, USA); rabbit anti-Sir2 alpha (Upstate Cell Signaling Solutions, Lake Placid, NY, USA); mouse anti-α-tubulin (Sigma). Secondary antibodies labeled with horseradish peroxidase were used as described (33).

Deacetylase activity assay using crude lysate

Recombinant GST-SIRT1 was expressed in *E. coli* BL21 (DE3) and pulled down with Glutathione Sepharose 4B (GE Healthcare, Buckinghamshire, UK). After washing the resin with wash buffer (50 mM Tris-HCl [pH 7.4], 0.1% Triton X-100, 300 mM NaCl) three times, GST-SIRT1 was eluted with glutathione buffer (20 mM Tris-HCl [pH 8.0], 10 mM glutathione, 100 mM NaCl, 0.01% Triton X-100); and the resulting solution was applied to an Ultrafree Filter (10K NMWL membrane; Millipore, Milford, MA, USA) for concentration and desalination. HEK293T cells (ϕ60 mm) were transfected with 2 μg of pEGFP-p53, incubated for 24 h, and treated with 1 μM actinomycin D (Sigma) and 0.5 μM TSA (Sigma). After an additional incubation for 18 h, the cells were lysed with lysis buffer (50 mM Tris-HCl [pH 7.4], 0.5% Triton X-100, 150 mM NaCl, 0.5 μM TSA) containing protease inhibitor cocktail (Roche, Basel, Switzerland). Following a brief sonication and centrifugation (20,000 × *g*, 10 min), 1 μg/μL cell lysate was incubated at 30°C for 60 min with 0.2 μg/μl GST-SIRT1, 400 μM NAD (Sigma), and 500 μM of the test compound. The samples were added to SDS sample buffer and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Immobilin-P membranes (Millipore). Signals were detected with ECL reagents (GE Healthcare).

Measurement of cell growth

HEK293T cells (5.0×10^3/well of 96-well plates) were incubated for 24 h, treated with 2, 6, or 20 μM of compound 17 and then incubated for 48 h. Measurement of cell growth was carried out by *WST-8* assay based on measurement of intracellular dehydrogenase activity. The cells were mixed with 1/10 volume of *WST-8* reagent (Nacalai Tesque), and the optical absorption was measured at a wavelength of 450 nm. The absorption intensity in the well without cells was subtracted from the values of the samples measured in the assay. The value of the samples in absence of drugs was used as the baseline.

Statistical analyses

Statistical significances among values from more than two groups (kinds of compounds in Fig. 2 or concentration of compounds in Figs. 3 and 4) were determined by a one-way analysis of variance (ANOVA, $P<0.05$) followed by Fisher's least-squares difference (FLD) test. When comparing two groups (20 μM of compound 17 and sirtinol in Figs. 3 and 4 or 0 and 20 μM of sirtinol in Fig. 4B), Student's *t*-test was used for statistical analysis.

Results

Screening of compounds inhibiting the SIRT1 deacetylase activity to p53 peptide

To search for activators or inhibitors of SIRT1, 36 polyphenols were purchased or synthesized (Fig. 1). They are classified into four derivative groups: stilbenes, chalcones, flavanones, and flavonols. Compounds 17 and 18 were chalcones synthesized here for the first time, while some known activators such as resveratrol and piceatannol (compound 1 and 2, respectively) were also included in the library. These 36 compounds were examined for deacetylase activity in vitro using a commercially available assay. In this assay, purified recombinant SIRT1, NAD, and the compound being tested were mixed with a substrate p53 peptide coupled to fluorophore and quencher. The degree of deacetylation was assessed by measuring the fluorescence intensity. Although some compounds, such as compounds 2 and 6, activated the deacetylation of SIRT1, the increase in deacetylation was only several-fold (Table 1). On the other hand, compounds 11, 17, and 18 suppressed the deacetylase activity of SIRT1 more than 90%, so we focused on the inhibitory effect of these compounds for further analysis.

Effect of the compounds on the deacetylation of p53 protein by SIRT1

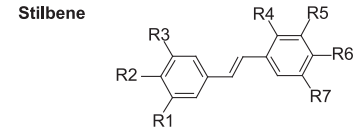
In the screening described above, p53 acetylated peptide was used as a substrate. We examined whether the candidates for SIRT1 inhibitors also suppressed deacetylation of p53 protein synthesized in cells. In the assay using crude cell lysate, sirtinol inhibited the SIRT1 deacetylase activity (Fig. 2). Among the candidates tested, only compound 17 inhibited the deacetylation of p53 ($P<0.001$, FLD test; Fig. 2). This result indicates that compound 17 is a novel SIRT1 deacetylase inhibitor that could fit into the conformational space between SIRT1 and full length p53.

Effect of compound 17 on the deacetylase activity of SIRT1 and SIRT3

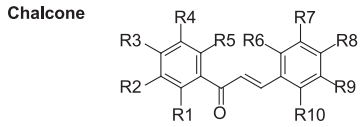
IC_{50} values of compound 17 against SIRT1 and SIRT3 activities were calculated based on the assay as described in Table 1. Compound 17 slightly preferred to inhibit SIRT1 rather than SIRT3 (Table 2). In addition, compound 17 was more active to either sirtuin than sirtinol ($P<0.01$ for either sirtuin, two-tailed Student's *t*-test). These results show that compound 17 is a potent inhibitor of sirtuins.

Effect of compound 17 on endogenous p53 in intact cells

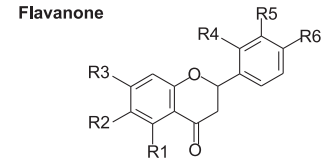
To examine the effect of compound 17 on the acetyla-



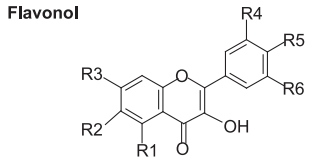
	R1	R2	R3	R4	R5	R6	R7
1	OH		OH			OH	
2	OH		OH			OH	OH
3		OH					
4	OH						
5		OH				OH	
6		OH					OCH_3
7				OH			
8					OH		OH
9					OH		OCH_3
10	OCH_3		OCH_3			OH	



	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
11	OH		OH					OH	OH	
12			OH		OH			OH		
13	OH									
14										OH
15			OH							
16								OH		
17			OH	OH	OH		OH			
18		OH			OH	OH		OH		OH



	R1	R2	R3	R4	R5	R6
19	OH		OH			OH
20	OH		OCH_3			
21		OH				
22			OH			
23				OH		
24						OH
25					OH	
26	OH		OH		OH	OCH_3
27	OH		OH		OH	OH
28	OH		OH			OH



	R1	R2	R3	R4	R5	R6
29			OH		OH	OH
30	OH		OH		OH	OH
31						
32		OH				
33			OH			
34	OH		OH	OH		OH
35	OH		OH	OH	OH	OH
36		OH		OH	OH	

Fig. 1. Chemical structures of the compounds used for the deacetylation assay in vitro. The compounds used in the screening were classified into four structural groups: stilbenes, chalcones, flavanones, and flavonols. Compounds 17 and 18 were newly synthesized by us. A blank indicates "H".

Table 1. Effect of chemical compounds on the deacetylase activity of SIRT1

Compound	SIRT1 deacetylase activity[a] (average ± S.E.M.)	Compound	SIRT1 deacetylase activity[a] (average ± S.E.M.)
–	1	19	1.27 ± 0.55
1	1.29 ± 0.10	20	1.48 ± 0.62
2	3.09 ± 0.75	21	1.50 ± 0.50
3	1.45 ± 0.42	22	1.60 ± 0.54
4	1.37 ± 0.03	23	1.39 ± 0.50
5	0.82 ± 0.07	24	0.64 ± 0.22
6	2.84 ± 0.07	25	1.03 ± 0.08
7	2.81 ± 0.19	26	1.17 ± 0.24
8	1.33 ± 0.22	27	1.45 ± 0.53
9	1.26 ± 0.03	28	1.36 ± 0.64
10	1.86 ± 0.05	29	1.39 ± 0.54
11	0.04 ± 0.03	30	0.38 ± 0.14
12	0.32 ± 0.08	31	0.84 ± 0.06
13	1.66 ± 0.52	32	0.64 ± 0.24
14	1.42 ± 0.50	33	1.32 ± 0.56
15	1.08 ± 0.56	34	0.88 ± 0.03
16	1.25 ± 0.41	35	0.43 ± 0.01
17	0.06 ± 0.01	36	0.25 ± 0.15
18	0.01 ± 0.01		

The reaction was performed at 25°C for 30 min in presence of 100 μM of the test compound. [a]The deacetylation level in the absence of a compound was taken as the baseline value of deacetylation. Data represent the mean values of at least three experiments.

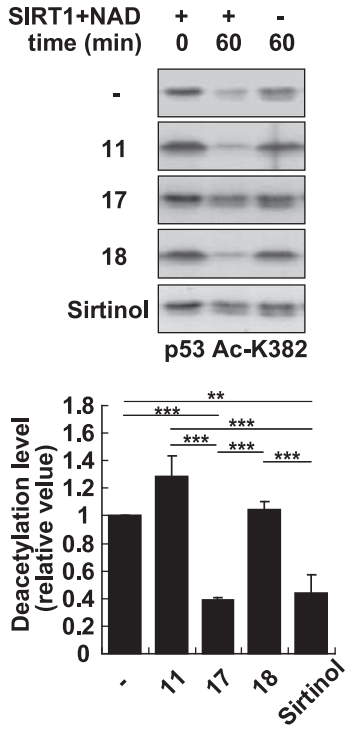


Fig. 2. Effect of the candidates for SIRT1 inhibitor on the deacetylation of p53 by SIRT1 in crude cell lysate. The acetylation of p53 was detected with anti-p53 acetylated K382 antibodies (upper panel), and quantitative analysis was carried out (bottom graph). The deacetylation level in the absence of a compound was taken as the baseline value of deacetylation. The experiments were repeated three times (FLD test, $^{**}P<0.01$, $^{***}P<0.001$).

Table 2. Inhibitory effect against SIRT1 and SIRT3

Compound	IC_{50} (μM) ± S.E.M.[a]	
	SIRT1	SIRT3
Compound 17	40.3 ± 2.8	48.0 ± 4.3
Sirtinol	120.2 ± 15.9	189.0 ± 22.2

[a]The reaction was performed at 25°C for 15 min. Experiments were repeated three times.

tion level of p53 in intact cells, HEK293T cells were treated with compound 17. Because the IC_{50} in the HDACs-activity assay was 445 ± 28 μM, low doses of compound 17 (2, 6, and 20 μM) were used in this assay to reduce the possibility that the compound affects other HDACs. Hyperacetylation of p53 was detected in the cells treated with compound 17, and this effect was dependent on the concentration of compound 17 used ($P<0.001$, FLD test; Fig. 3). Furthermore, the level of acetylated p53 was higher for compound 17 than sirtinol. Therefore, compound 17 is a novel potent inhibitor of SIRT1 deacetylation in intact cells.

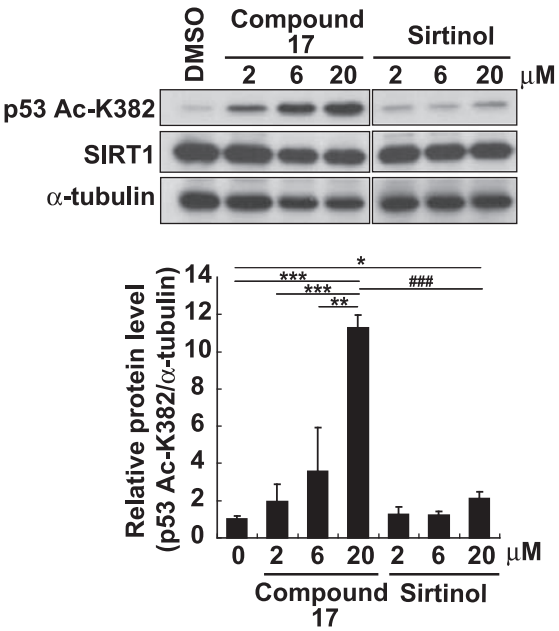


Fig. 3. Effect of compound 17 on the acetylation level of p53 in intact cells. HEK293T cells were treated with 2, 6, or 20 μM of compound 17 for 20 h. A 10-μg sample of total cell lysate was used for the analysis. Quantitative analysis was performed (bottom graph). The experiments were repeated four times (FLD test, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; two-tailed Student's *t*-test, $^{\#\#\#}P<0.001$).

Effect of compound 17 on the protein level of p21$^{CIP1/WAF1}$ and cell growth

It has been shown that the overexpression of SIRT1 suppresses the expression of $p21^{CIP1/WAF1}$, a cyclin-dependent kinase (CDK) inhibitor-1A that is known to arrest cell cycle, and that the dominant negative mutant of SIRT1 (hSir2HY) increases the protein level of $p21^{CIP1/WAF1}$ after ionizing radiation (13, 16, 34). Therefore, we examined the protein level of $p21^{CIP1/WAF1}$ in the cells treated with compound 17. The level of $p21^{CIP1/WAF1}$ was higher in the samples treated with 20 μM of compound 17 than non-treated samples ($P<0.05$, FLD test; Fig. 4A). While the accumulation of $p21^{CIP1/WAF1}$ was slightly increased at 20 μM sirtinol, it was clear that compound 17 had a more pronounced effect on the accumulation of $p21^{CIP1/WAF1}$ than sirtinol at the same concentration. Furthermore, the cell growth of HEK293T cells was suppressed by the treatment of 20 μM compound 17 compared to no treatment ($P<0.001$, FLD test; Fig. 4B). Therefore, our results show that compound 17 (3,2',3',4'-tetrahydroxychalcone) suppresses cell proliferation.

Discussion

Posttranslational modifications are key events during the physiological regulation of protein. In our previous

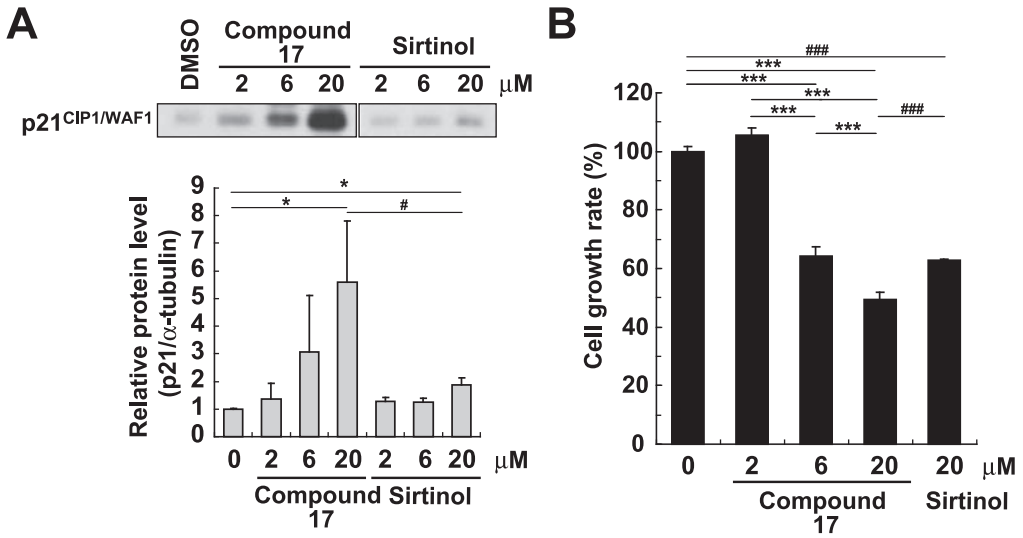


Fig. 4. Effect of compound 17 on cell growth. A: $p21^{CIP1/WAF1}$ protein was detected in the samples used in Fig. 3. B: cell growth rate of the HEK293T cells was measured with the *WST-8* assay. The cells were incubated for 48 h after the treatment with compound 17. The experiments were repeated four times (A) or five times (B). Statistical analysis was carried out by the FLD test (**P*<0.05, ****P*<0.001) or two-tailed Student's *t*-test ($^{\#}P$<0.05, $^{\#\#\#}P$<0.001).

reports, we studied the phosphorylation, ubiquitination, sumoylation, and polyglutamylation of proteins. While gene targeting, mutagenesis, and RNAi technologies were used in those studies to modulate the enzymatic activity, they can not be always suitable for analysis of a posttranslational modification. In this study, we focused on a pharmacological approach and identified compound 17 as a potent SIRT1 inhibitor that at least acts on the deacetylation of p53. This finding is expected to contribute to the studies on deacetylation by sirtuins or p53-mediated physiological effects.

Resveratrol (compound 1) and its analogues piceatannol (compound 2), butein (compound 11), isoliquiritigenin (compound 12), fisetin (compound 29), and quercetin (compound 30) have been reported to activate the deacetylase activity of sirtuin from 4.6- to 13.4-fold (1). In the present study, however, resveratrol and fisetin exhibited only marginal activation of the SIRT1 deacetylase activity (1.29- and 1.39-fold, respectively), while piceatannol showed the most powerful activation of the tested compounds (3.09-fold, Table 1). Butein, isoliquiritigenin, and quercetin suppressed the deacetylase activity (0.04-, 0.32-, and 0.38-fold, respectively). These different results with the previous data were thought to be due to the characteristics of the substrate peptide used here. A quencher prevents fluorophore from emitting fluorescence when it is in close enough proximity. In this assay system, a substrate that has a quencher and a fluorophore conjugated at opposite ends was used. Since it is known that the proteolysis was tightly followed by deacetylation by SIRT1 in this system, the extent of changes that reflect the detachment of quencher and fluorophore detected by a fluorometer was used as a parameter for SIRT1 activity. Because it was suggested that the increased SIRT1 activity induced by resveratrol in the previous assay was caused by substrate-specific activation (35, 36), our results would seem to be the result of improving the construction of the substrate.

Several hydrogen-bond donors, for example, hydroxyl groups, have been suggested to be important for an inhibitor to interact with a putative SIRT2 active site (29, 30). As the site is highly conserved in SIRT1, it is assumed that there are some polyphenols where the location of the hydroxyl groups is potentially suitable for the interaction with SIRT1 at the putative active site. Here, we showed that compounds 11, 17, and 18 greatly inhibited the deacetylation by SIRT1 in the assay using p53 peptide (Table 1). Those compounds have common features: they belong to the chalcone polypenols; and they have more than four hydroxyl groups (Fig. 1). Therefore, both of those features seem to be necessary for the potent inhibitory effect in the assay. On the other hand, compounds 11 and 18 did not inhibit the deacetylation of full length p53 (Fig. 2). This result would be caused by the conformational difference of the SIRT1-substrates complex. It is thought that compounds 11 and 18 can freely access SIRT1 bound to p53 peptide but not to full length p53 that is buried in the access space. In any case, it is clear that compound 17 is effective for inhibiting SIRT1-mediated deacetylation in the presence of either substrate.

Sirtinol indubitably showed the inhibitory effect on the deacetylase activity of SIRT1 in our report. The IC_{50} of sirtinol to SIRT1 in the assay using the p53 peptide was 120.2 μM (Table 2). This result is consistent with the previous data (131 μM) from a different fluorimetric assay (26). On the other hand, the IC_{50} of compound 17 against SIRT1 was 40.3 μM and lower than that of sirtinol. The tendency for compound 17 to be more effective than sirtinol can be also be seen in Figs. 3 and 4. While some compounds showing much lower IC_{50} values to SIRT1 have been reported (27), it is interesting that polyphenols, many of which have been regarded to activate sirtuins, can show the opposite effect and that additional inhibitors might be found among the the polyphenols in the future.

Compound 17 increased the protein level of $p21^{CIP1/WAF1}$ and suppressed cell proliferation (Fig. 4). This result is in agreement with a previous report that $p21^{CIP1/WAF1}$ inhibits CDK activity and contributes to cell cycle arrest (34) and that some sirtuin inhibitors induce senescence-like growth arrest in cancer cells (31, 32). Some transcriptional factors, including p53, are known to function in the $p21^{CIP1/WAF1}$ promoter (37). Therefore, such transcriptional factors would be involved with the increase of $p21^{CIP1/WAF1}$ in the cells treated with compound 17. Especially, p53 is regarded to be a primary candidate because p53 is a target for the deacetylation by SIRT1, and in fact, acetylated p53 was increased in the cell treated with compound 17 (Fig. 3). Alternatively, histone would be implicated in the transcriptional activation of $p21^{CIP1/WAF1}$ in the presence of compound 17 because it is also a target for the deacetylation by SIRT1. Sirtinol showed a similar effect on cell proliferation as compound 17 despite causing only a marginal increase of the protein level of $p21^{CIP1/WAF1}$ and acetylated p53 (Figs. 3 and 4). This result suggests the possibility that sirtinol induces cell death or suppresses cell proliferation independent of inhibiting the deacetylation by SIRT1 at the indicated concentration.

In conclusion, we show that a novel chalcone, 3,2',3',4'-tetrahydroxychalcone, inhibits the deacetylation by SIRT1 and suppresses the cell growth. Because it has been reported that cambinol and sirtinol, sirtuin inhibitors, can exert anticancer effect on human cancer cells (31, 32), it would be well worth applying compound 17 or its derivatives to not only basic studies, but also therapeutic applications for anticancer in the future.

Acknowledgments

We thank Dr. S. Imai for providing the SIRT1 cDNA clone. We also thank K. Matsuda (Mitsubishi Chemical Corporation) and M. Takai (Mitsubishi Chemical Group Science and Technology Research Center) for their helpful advice. We appreciate the valuable input and technical assistance of Dr. S. Asai, K. Ohtsu, and M. Arai and other colleagues at the Mitsubishi Kagaku Institute of Life Sciences.

References

1 Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, et al. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature. 2003;425:191–196.
2 Kaeberlein M, McVey M, Guarente L. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev. 1999;13:2570–2580.
3 Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, et al. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature. 2004;430:686–689.
4 Tissenbaum HA, Guarente L. Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. Nature. 2001;410: 227–230.
5 Wang C, Wang MW, Tashiro S, Onodera S, Ikejima T. Roles of SIRT1 and phosphoinositide 3-OH kinase/protein kinase C pathways in evodiamine-induced human melanoma A375-S2 cell death. J Pharmacol Sci. 2005;97:494–500.
6 Hisahara S, Chiba S, Matsumoto H, Horio Y. Transcriptional regulation of neuronal genes and its effect on neural functions: NAD-dependent histone deacetylase SIRT1 (Sir2alpha). J Pharmacol Sci. 2005;98:200–204.
7 Guarente L, Picard F. Calorie restriction – the SIR2 connection. Cell. 2005;120:473–482.
8 Kahyo T, Mostoslavsky R, Goto M, Setou M. Sirtuin-mediated deacetylation pathway stabilizes Werner syndrome protein. FEBS Lett. 2008;582:2479–2483.
9 Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature. 2000;403:795–800.
10 Blander G, Guarente L. The Sir2 family of protein deacetylases. Annu Rev Biochem. 2004;73:417–435.
11 de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB. Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J. 2003;370:737–749.
12 Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science. 2004;303:2011–2015.
13 Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, et al. Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell. 2001;107:137–148.
14 Motta MC, Divecha N, Lemieux M, Kamel C, Chen D, Gu W, et al. Mammalian SIRT1 represses forkhead transcription factors. Cell. 2004;116:551–563.
15 Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature. 2005;434:113–118.
16 Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, et al. hSIR2(SIRT1) functions as an NAD-dependent p53

deacetylase. Cell. 2001;107:149–159.
17 Lavin MF, Gueven N. The complexity of p53 stabilization and activation. Cell Death Differ. 2006;13:941–950.
18 Wu Z, Wu L, Li L, Tashiro S, Onodera S, Ikejima T. p53-mediated cell cycle arrest and apoptosis induced by shikonin via a caspase-9-dependent mechanism in human malignant melanoma A375-S2 cells. J Pharmacol Sci. 2004;94:166–176.
19 Aylon Y, Oren M. Living with p53, dying of p53. Cell. 2007; 130:597–600.
20 Jackson MD, Schmidt MT, Oppenheimer NJ, Denu JM. Mechanism of nicotinamide inhibition and transglycosidation by Sir2 histone/protein deacetylases. J Biol Chem. 2003;278: 50985–50998.
21 Sauve AA, Schramm VL. Sir2 regulation by nicotinamide results from switching between base exchange and deacetylation chemistry. Biochemistry. 2003;42:9249–9256.
22 Schmidt MT, Smith BC, Jackson MD, Denu JM. Coenzyme specificity of Sir2 protein deacetylases: implications for physiological regulation. J Biol Chem. 2004;279:40122–40129.
23 Bedalov A, Gatbonton T, Irvine WP, Gottschling DE, Simon JA. Identification of a small molecule inhibitor of Sir2p. Proc Natl Acad Sci U S A. 2001;98:15113–15118.
24 Grozinger CM, Chao ED, Blackwell HE, Moazed D, Schreiber SL. Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. J Biol Chem. 2001;276:38837–38843.
25 Hirao M, Posakony J, Nelson M, Hruby H, Jung M, Simon JA, et al. Identification of selective inhibitors of NAD+-dependent deacetylases using phenotypic screens in yeast. J Biol Chem. 2003;278:52773–52782.
26 Mai A, Massa S, Lavu S, Pezzi R, Simeoni S, Ragno R, et al. Design, synthesis, and biological evaluation of sirtinol analogues as class III histone/protein deacetylase (Sirtuin) inhibitors. J Med Chem. 2005;48:7789–7795.
27 Napper AD, Hixon J, McDonagh T, Keavey K, Pons JF, Barker J, et al. Discovery of indoles as potent and selective inhibitors of the deacetylase SIRT1. J Med Chem. 2005;48:8045–8054.
28 Posakony J, Hirao M, Stevens S, Simon JA, Bedalov A. Inhibitors of Sir2: evaluation of splitomicin analogues. J Med Chem. 2004;47:2635–2644.
29 Tervo AJ, Kyrylenko S, Niskanen P, Salminen A, Leppanen J, Nyronen TH, et al. An in silico approach to discovering novel inhibitors of human sirtuin type 2. J Med Chem. 2004;47:6292–6298.
30 Trapp J, Jochum A, Meier R, Saunders L, Marshall B, Kunick C, et al. Adenosine mimetics as inhibitors of NAD+-dependent histone deacetylases, from kinase to sirtuin inhibition. J Med Chem. 2006;49:7307–7316.
31 Heltweg B, Gatbonton T, Schuler AD, Posakony J, Li H, Goehle S, et al. Antitumor activity of a small-molecule inhibitor of human silent information regulator 2 enzymes. Cancer Res. 2006;66:4368–4377.
32 Ota H, Tokunaga E, Chang K, Hikasa M, Iijima K, Eto M, et al. Sirt1 inhibitor, Sirtinol, induces senescence-like growth arrest with attenuated Ras-MAPK signaling in human cancer cells. Oncogene. 2006;25:176–185.
33 Yao I, Takagi H, Ageta H, Kahyo T, Sato S, Hatanaka K, et al. SCRAPPER-dependent ubiquitination of active zone protein RIM1 regulates synaptic vesicle release. Cell. 2007;130:943–957.
34 Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell. 1993;75:805–816.
35 Borra MT, Smith BC, Denu JM. Mechanism of human SIRT1 activation by resveratrol. J Biol Chem. 2005;280:17187–17195.
36 Kaeberlein M, McDonagh T, Heltweg B, Hixon J, Westman EA, Caldwell SD, et al. Substrate-specific activation of sirtuins by resveratrol. J Biol Chem. 2005;280:17038–17045.
37 Gartel AL, Tyner AL. Transcriptional regulation of the p21((WAF1/CIP1)) gene. Exp Cell Res. 1999;246:280–289.