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Structural requirements of flavonoids for the adipogenesis of 3T3-L1 cells

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ABSTRACT

To search for a new class of antidiabetic compounds, effects of 44 flavonoids on the adipogenesis of 3T3-L1 cells were examined. Among them, 3,4′,7-trimethylkaempferol, tetramethylkaempferol, and pentamethylquercetin concentration-dependently enhanced the accumulation of triglyceride, a marker of adipogenesis. With regard to structural requirements of flavonoids for the activity, it was fond that: (1) most flavonoids having hydroxy groups lacked the effect; (2) flavonols with methoxy groups showed stronger effects particularly those with a methoxy group at the 3-position; and (3) a methoxy group of flavonols at the B ring was also important. 3,4′,7-Trimethylkaempferol, tetramethylkaempferol, and pentamethylquercetin significantly increased the amount of adiponectin released into the medium and the uptake of 2-deoxyglucose into the cells. Furthermore, tetramethylkaempferol and pentamethylquercetin also increased mRNA levels of adiponectin, glucose transporter 4 (GLUT4), and fatty acid-binding protein (aP2). Both compounds also increased the mRNA levels of peroxisome proliferator-activated receptor (PPAR)γ2 and CCAAT/enhancer-binding protein (C/EBP)α, β, and/or δ, although, different from troglitazone, they did not activate PPARγ directly in a nuclear receptor cofactor assay.

1. Introduction

In modern society, diabetes mellitus is a chronic and often undiagnosed or inadequately treated disease. Type 2 diabetes is closely associated with other metabolic disorders, such as hypertension, cardiovascular diseases, and atherosclerosis and its incidence is increasing worldwide. Insulin resistance is an important marker for developing type 2 diabetes. The roles of life-style changes and weight loss in preventing diabetes have been proven in clinical trials. Several oral hypoglycemic agents and the antiobese drug orlistat have been shown to significantly decrease progression to diabetes. However, more effective and safe medicines are required.[1]

Previous studies have demonstrated that flavonoids and/or flavonoid-rich fractions ameliorate experimental diabetes in mice and rats.[2–4] We also reported inhibitory activities of various flavonoids against aldose reductase, a key enzyme in the polyol pathway, and the formation of advanced glycation end-products (AGEs) in vitro, and clarified several structural requirements for the activity.[5,6] However, several flavonoids were reported to inhibit insulin signals in adipocytes.[7,8]

Thiazolidinedione-type compounds such as pioglitazone are major ligands of peroxisome proliferator-activated receptor γ (PPARγ) and potent insulin sensitizers, and increase the levels of adiponectin, an important adipocytokine associated with insulin sensitivity in adipose tissue. These compounds are currently used clinically to treat type 2 diabetes.[9–12] It is well established that PPARγ agonists such as thiazolidinedione-type compounds promote the adipogenesis of 3T3-L1 cells, and so the cells have been used for the development of anti-diabetic compounds.[13–18]

In this study, we examined the effects of 44 flavonoids (Figs. 1 and 2) on the adipogenesis of 3T3-L1 cells in order to find PPARγ agonist-like compounds, and mode of action of the active compounds was investigated.

2. Results and discussion

2.1. Effects on triglyceride accumulation in the cells

As shown in Table 1, a PPARγ agonist, troglitzazone, promoted the accumulation of triglyceride (TG), as a marker of adipogenesis, in the cells with a maximum effect observed at 1–3 μM. Among the compounds tested, only 3,4′,7-trimethylkaempferol (**26**), tetramethylkaempferol (**30**), and pentamethylquercetin (**31**) promoted the accumulation in a concentration-dependent manner at 1–30 μM.

Cytotoxic effects of flavonoids have been reported in many cell lines.[19–21] In the present study, the marked reduction in TG levels observed on treatment with several flavonoids (e.g. **8**, **12**, **13**, **14**, **17**, and **44**) at a higher concentration (30 μM) may depend on cyto-

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Table 1
Effects of flavonoids (**1**–**44**) on TG levels in 3T3-L1 cells

	Increase (%) in TG levels Concn (μM)					
	0	0.3	1	3	10	30
Flavone						
Flavone (**1**)	0.0 ± 1.8	—	−2.6 ± 1.7	−6.8 ± 4.1	−5.2 ± 2.6	−10.9 ± 3.7*
3′,4′-Dihydroxyflavone (**2**)	0.0 ± 4.0	—	5.9 ± 3.4	13.8 ± 2.8*	9.8 ± 4.4	−21.0 ± 2.2**
7-Hydroxyflavone (**3**)	0.0 ± 2.8	—	12.2 ± 9.1	13.7 ± 8.7	17.1 ± 1.5	5.3 ± 5.6
4′,7-Dihydroxyflavone (**4**)	0.0 ± 5.3	—	−8.1 ± 2.3	−3.9 ± 3.6	−1.5 ± 5.5	−5.7 ± 5.2
3′,4′,7-Trihydroxyflavone (**5**)	0.0 ± 2.8	—	4.5 ± 2.5	9.0 ± 3.1	14.8 ± 4.1*	−1.7 ± 5.8
4′,7-Dimethoxyflavone (**6**)	0.0 ± 2.8	—	1.2 ± 5.0	3.4 ± 2.5	4.3 ± 5.1	6.3 ± 2.7
Apigenin (**7**)	0.0 ± 5.5	—	4.5 ± 4.5	4.9 ± 6.3	7.0 ± 4.4	−7.7 ± 7.2
Luteolin (**8**)	0.0 ± 2.8	—	3.9 ± 4.0	−6.4 ± 3.8	−14.2 ± 5.8	−41.3 ± 6.4**,[a]
Wogonin (**9**)	0.0 ± 4.4	—	7.8 ± 5.4	9.6 ± 5.5	19.2 ± 2.9*	16.6 ± 4.2*
Tectochrysin (**10**)	0.0 ± 1.7	—	7.4 ± 3.7	10.7 ± 4.2	10.4 ± 1.2	17.5 ± 7.7
4′,7-Dimethylapigenin (**11**)	0.0 ± 5.0	7.7 ± 5.3	19.0 ± 1.5*	12.2 ± 6.1	5.8 ± 2.8	6.8 ± 8.8
Baicalein (**12**)	0.0 ± 2.7	—	−5.2 ± 2.4	−7.7 ± 5.0	−21.8 ± 3.6**	−37.2 ± 2.3**,[a]
5,7-Dimethoxyflavone (**13**)	0.0 ± 2.5	—	7.5 ± 3.7	3.4 ± 6.5	6.3 ± 4.1	−88.1 ± 4.5**,[a]
Trimethylapigenin (**14**)	0.0 ± 4.9	5.6 ± 6.5	16.7 ± 1.7*	14.1 ± 3.3*	−17.2 ± 6.0*	−83.0 ± 1.4**,[a]
Tetramethylluteolin (**15**)	0.0 ± 5.2	—	2.6 ± 5.9	3.1 ± 4.5	7.4 ± 3.7	−15.3 ± 5.6
Nobiletin (**16**)	0.0 ± 4.1	14.1 ± 5.4	15.3 ± 6.3*	12.6 ± 2.8	10.7 ± 3.0	−6.7 ± 3.4
Flavonol						
Flavonol (**17**)	0.0 ± 2.9	—	−12.6 ± 2.8	−10.0 ± 2.5	−17.6 ± 5.1**	−31.6 ± 3.2**,[a]
Fisetin (**18**)	0.0 ± 4.9	—	4.1 ± 4.8	−2.4 ± 4.9	−8.8 ± 3.3	−2.7 ± 3.9
Kaempferol (**19**)	0.0 ± 3.4	—	6.2 ± 3.5	5.9 ± 4.9	0.3 ± 3.4	3.7 ± 4.3
Quercetin (**20**)	0.0 ± 3.5	—	7.0 ± 6.1	5.8 ± 2.4	3.0 ± 6.0	3.9 ± 4.9
Myricetin (**21**)	0.0 ± 2.9	—	4.3 ± 1.7	2.7 ± 4.2	−0.7 ± 4.5	5.6 ± 3.2
Gossypetin (**22**)	0.0 ± 3.2	—	17.5 ± 1.6	−2.9 ± 7.6	−6.7 ± 6.6	−9.5 ± 3.0
Rhamnetin (**23**)	0.0 ± 2.4	15.5 ± 5.4	19.0 ± 4.5**	1.9 ± 2.2	2.4 ± 5.8	−21.4 ± 4.9**
Ombuine (**24**)	0.0 ± 3.3	—	−2.1 ± 2.8	5.9 ± 1.7	4.6 ± 1.8	24.7 ± 2.4**
5-Hydroxy-3,7-dimethoxyflavone (**25**)	0.0 ± 3.7	—	13.3 ± 3.4	13.7 ± 0.5	14.2 ± 2.8	17.7 ± 6.1*
3,4′,7-Trimethylkaempferol (**26**)	0.0 ± 3.6	9.3 ± 4.3	21.7 ± 0.4**	20.8 ± 2.7**	22.5 ± 1.3**	23.0 ± 1.6**
Ayanin (**27**)	0.0 ± 6.6	—	2.6 ± 4.4	16.3 ± 5.6	18.5 ± 5.5	0.4 ± 8.3
Retusine (**28**)	0.0 ± 3.4	—	3.8 ± 5.7	11.2 ± 2.9	21.0 ± 2.8*	3.8 ± 6.2
3,5,7-Trimethoxyflavone (**29**)	0.0 ± 3.0	—	9.0 ± 5.5	10.4 ± 3.3	13.2 ± 2.7	6.6 ± 6.9
Tetramethylkaempferol (**30**)	0.0 ± 5.1	10.1 ± 4.7	19.1 ± 5.2*	24.1 ± 6.6*	28.2 ± 5.8**	33.7 ± 4.5**
Pentamethylquercetin (**31**)	0.0 ± 2.8	9.4 ± 2.9	17.7 ± 2.4**	26.2 ± 3.2**	27.0 ± 3.6**	27.4 ± 2.1**
Kaempferol 3-O-β-D-glucopyranoside (**32**)	0.0 ± 0.8	—	2.1 ± 3.7	4.7 ± 4.9	8.8 ± 7.2	10.2 ± 5.3
Quercetin 3-*O*-β-D-glucopyranoside (**33**)	0.0 ± 3.1	—	−2.2 ± 3.3	0.1 ± 5.1	−0.2 ± 1.2	−2.4 ± 2.7
Flavanone						
Flavanone (**34**)	0.0 ± 1.6	—	−1.9 ± 5.2	5.1 ± 2.9	2.5 ± 3.2	1.0 ± 7.4
7-Methylliquiritigenin (**35**)	0.0 ± 2.4	—	−1.2 ± 2.1	2.2 ± 2.7	2.6 ± 2.4	13.5 ± 2.6
Eriodictyol (**36**)	0.0 ± 3.8	—	1.6 ± 0.5	−5.0 ± 1.5	−10.3 ± 2.4	−10.3 ± 7.0
Flavanol (catechin)						
(+)-Catechin (**37**)	0.0 ± 1.8	—	−0.5 ± 4.5	6.4 ± 11.6	7.9 ± 9.8	11.9 ± 6.2
(−)-Epicatechin (**38**)	0.0 ± 8.2	—	3.7 ± 2.8	6.1 ± 5.2	−7.2 ± 9.1	−0.6 ± 9.3
(−)-Epigallocatechin (**39**)	0.0 ± 1.3	—	−9.5 ± 6.0	−3.8 ± 1.8	−10.2 ± 4.0	−19.5 ± 2.2**
Isoflavone						
Daidzein (**40**)	0.0 ± 4.5	—	2.0 ± 3.0	6.5 ± 6.3	8.5 ± 4.3	13.4 ± 3.6
Genistein (**41**)	0.0 ± 2.1	—	−4.3 ± 0.4	−4.3 ± 3.1	−17.6 ± 9.2	−19.2 ± 4.7*
Biochanin A (**42**)	0.0 ± 1.5	—	2.6 ± 4.7	6.1 ± 9.8	1.2 ± 4.6	−16.5 ± 6.6
Chalcone						
Isoliquiritigenin (**43**)	0.0 ± 11.5	—	2.7 ± 10.8	4.0 ± 9.6	−0.7 ± 9.2	−33.7 ± 2.8*
Butein (**44**)	0.0 ± 3.3	—	14.8 ± 1.9	17.5 ± 7.0	−7.3 ± 6.8	−80.8 ± 4.1**,[a]
Troglitazone	0.0 ± 4.1	31.9 ± 1.6=	35.8 ± 6.0**	34.6 ± 2.4**	28.1 ± 2.0**	—

Refer to Section 4.3 for the experimental methods. Briefly, 3T3-L1 cells (5.0×10^4 cells/well) in DMEM supplemented with 10% FBS were seeded into a 48-well multiplate. After 24 h, differentiation was induced by changing the medium to a differentiation medium [DMEM (high glucose) supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 5 μg/mL insulin]. After 3 d, the differentiation medium was replaced with a maintenance medium [DMEM (high glucose) supplemented with 10% FBS and 5 μg/mL insulin]. After 4 d (on day 8), the medium was removed and H_2O (200 μL/well) was added to each well, and then the cells were sonicated. The triglyceride (TG) level in the sonicate was determined. The test compound dissolved in DMSO was added to the differentiation and maintenance media. Values represent the mean ± SEM of the percent increase in TG levels ($n = 4$). Significantly different from the control group, $^*p < 0.05$, $^{**}p < 0.01$.

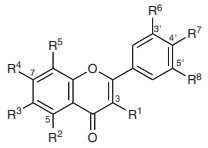
[a] The cells tended to detach from the surface of the culture plate.

toxic effects, since the cells tended to detach from the surface of the culture plate at the higher concentration, although further mechanisms such as the inhibition of insulin signal transduction or cell adhesion mechanisms should be examined. In addition, Saito et al. reported that a methoxyflavone, nobiletin (**16**), enhanced differentiation and lipolysis in 3T3-L1 adipocytes.[3] In the present study, **16** caused little accumulation of TG at 1–10 μM under our experimental conditions.

With regard to structural requirements of flavonoids for the activity, it was found that: (1) most flavonoids having hydroxy groups lacked the effects; (2) flavonols with methoxy groups showed stronger effects particularly those with a methoxy group at the 3-position [increase in TG at 3 μM: **11** (12.2%) < **26** (20.8%), **14** (14.1%) < **30** (24.1%), **15** (3.1%) < **31** (26.2%)]; and (3) a methoxy group of flavonols at the B ring was also important [increase in TG at 3 μM: **25** (13.7%) < **26** (20.8%), **29** (10.4%) < **30** (24.1%) and **31** (26.2%)].

2.2. Effects on adiponectin release into the medium

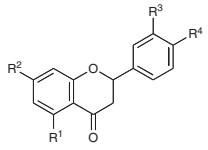
Adiponectin is an adipocyte-derived hormone that reverses insulin resistance associated with both lipoatrophy and obesity.[10] We therefore examined effects of three methoxyflavonols [3,4′,7-trimethylkaempferol (**26**), tetramethylkaempferol (**30**), and pentamethylquercetin (**31**)] on the release of adiponectin into the medium.



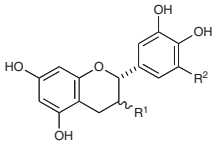
	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8
Flavone (**1**):	H	H	H	H	H	H	H	H
3',4'-Dihydroxyflavone (**2**):	H	H	H	H	H	OH	OH	H
7-Hydroxyflavone (**3**):	H	H	H	OH	H	H	H	H
4',7-Dihydroxyflavone (**4**):	H	H	H	OH	H	H	OH	H
3',4',7-Trihydroxyflavone (**5**):	H	H	H	OH	H	OH	OH	H
4',7-Dimethoxyflavone (**6**):	H	H	H	OCH_3	H	H	OCH_3	H
Apigenin (**7**):	H	OH	H	OH	H	H	OH	H
Luteolin (**8**):	H	OH	H	OH	H	OH	OH	H
Wogonin (**9**):	H	OH	H	OH	OCH_3	H	H	H
Tectochrysin (**10**):	H	OH	H	OCH_3	H	H	H	H
4',7-Dimethylapigenin (**11**):	H	OH	H	OCH_3	H	H	OCH_3	H
Baicalein (**12**):	H	OH	OH	OH	H	H	H	H
5,7-Dimethoxyflavone (**13**):	H	OCH_3	H	OCH_3	H	H	H	H
Trimethylapigenin (**14**):	H	OCH_3	H	OCH_3	H	H	OCH_3	H
Tetramethylluteolin (**15**):	H	OCH_3	H	OCH_3	H	OCH_3	OCH_3	H
Nobiletin (**16**):	H	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	H
Flavonol (**17**):	OH	H	H	H	H	H	H	H
Fisetin (**18**):	OH	H	H	OH	H	OH	OH	H
Kaempferol (**19**):	OH	OH	H	OH	H	H	OH	H
Quercetin (**20**):	OH	OH	H	OH	H	OH	OH	H
Myricetin (**21**):	OH	OH	H	OH	H	OH	OH	OH
Gossypetin (**22**):	OH	OH	H	OH	OH	OH	OH	H
Rhamnetin (**23**):	OH	OH	H	OCH_3	H	OH	OH	H
Ombuine (**24**):	OH	OH	H	OCH_3	H	OH	OCH_3	H
5-Hydroxy-3,7-dimethoxyflavone (**25**):	OCH_3	OH	H	OCH_3	H	H	H	H
3,4',7-Trimethylkaempferol (**26**):	OCH_3	OH	H	OCH_3	H	H	OCH_3	H
Ayanin (**27**):	OCH_3	OH	H	OCH_3	H	OH	OCH_3	H
Retusine (**28**):	OCH_3	OH	H	OCH_3	H	OCH_3	OCH_3	H
3,5,7-Trimethoxyflavone (**29**):	OCH_3	OCH_3	H	OCH_3	H	H	H	H
Tetramethylkaempferol (**30**):	OCH_3	OCH_3	H	OCH_3	H	H	OCH_3	H
Pentamethylquercetin (**31**):	OCH_3	OCH_3	H	OCH_3	H	OCH_3	OCH_3	H
Kaempferol 3-*O*-Glc (**32**):	O-Glc	OH	H	OH	H	H	OH	H
Quercetin 3-*O*-Glc (**33**):	O-Glc	OH	H	OH	H	OH	OH	H

Glc: β-D-glucopyranosyl

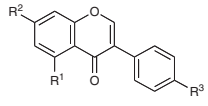
Figure 1. Chemical structures of flavones (**1**–**16**) and flavonols (**17**–**33**).



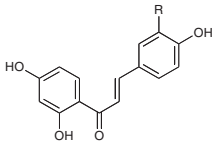
	R^1	R^2	R^3	R^4
Flavanone (**34**):	H	H	H	H
7-Methylliquiritigenin (**35**):	H	OCH_3	H	OH
Eriodictyol (**36**):	OH	OH	OH	OH



	R^1	R^2
(+)-Catechin (**37**):	β-OH	H
(−)-Epicatechin (**38**):	α-OH	H
(−)-Epigallocatechin (**39**):	α-OH	OH



	R^1	R^2	R^3
Daidzein (**40**):	H	OH	OH
Genistein (**41**):	OH	OH	OH
Biochanin A (**42**):	OH	OH	OCH_3



	R
Isoliquiritigenin (**43**):	H
Butein (**44**):	OH

Figure 2. Chemical structures of flavanones (**34**–**36**), flavan-3-ols (**37**–**39**), isoflavones (**40**–**42**), and chalcones (**43**, **44**).

As shown in Table 2, **30** and **31** increased adiponectin levels with a maximum effect at 10 and 3 μM, respectively, similar to troglitazone. But, **26** had a weaker effect.

2.3. Effects on uptake of 2-deoxyglucose

Next, effects of **26**, **30**, and **31** on the uptake of 2-deoxyglucose (2-DG) stimulated by insulin were examined. In cells incubated with the test compounds from the differentiated stage, **30** and **31** increased the uptake of 2-DG with a maximum effect at 3 μM, and again, **26** had a weak effect (Table 3).

Previously, Nomura et al. reported that flavonoids [e.g. apigenin (**7**), luteolin (**8**), fisetin (**18**), kaempferol (**19**), quercetin (**20**), genistein (**41**)] inhibited insulin signal transduction, and inhibited the translocation of GLUT4 and uptake of glucose in MC3T3-G2/PA6 adipose cells.[8] However, Fang et al. reported that kaempferol (**19**) and quercetin (**20**) improved glucose uptake in 3T3-L1 cells without adipogenic activity.[22] In the present study, consistent with the report by Nomura et al.,[8] kaempferol (**19**) concentration-dependently inhibited the uptake of 2-DG under the conditions that the compounds were incubated with insulin 20 min before the incubation with 2-DG in 3T3-L1 adipocytes (on day 8) (Table 3). Compounds **26**, **30**, and **31** also inhibited the uptake of 2-DG similar to **19**, which may contribute to their reduced effects on the uptake of 2-DG at higher concentrations.

Table 2
Effects of 3,4′,7-trimethylkaempferol (**26**), tetramethylkaempferol (**30**), and pentamethylquercetin (**31**) on the release of adiponectin into the medium

Concn (μM)	Adiponectin release (% of control)			
	3,4′,7-Trimethylkaempferol (**26**)	Tetramethylkaempferol (**30**)	Pentamethylquercetin (**31**)	Troglitazone
0 (DMSO)	100.0 ± 5.0	100.0 ± 3.4	100.0 ± 2.0*	100.0 ± 3.4
1	119.7 ± 5.6*	144.6 ± 1.7**	137.8 ± 4.6**	—
3	125.3 ± 1.7**	149.5 ± 1.7**	156.8 ± 6.3**	166.4 ± 2.0**
10	123.3 ± 6.5**	164.7 ± 4.6**	131.4 ± 4.6**	—
30	131.9 ± 3.3**	136.7 ± 4.1**	128.1 ± 6.4**	—

Refer to Section 4.4 for the experimental methods. Briefly, after the differentiation, the medium was replaced with the maintenance medium. On day 8, adiponectin concentrations in the medium were determined. The test compound dissolved in DMSO was added to the differentiation and maintenance media.
Values represent the mean ± SEM (n = 4). Significantly different from the control group, *p <0.05, **p <0.01.

2.4. Effects on mRNA expression of adiponectin, glucose transporter 4, fatty acid-binding protein, and CCAAT/enhancer-binding proteins

Next, effects of **30** and **31** on mRNA levels of adiponectin, glucose transporter 4 (GLUT4), and a fatty acid-binding protein (aP2) as a marker of adipogenesis, were examined. As shown in Table 4, both compounds increased mRNA levels of adiponectin, GLUT4, and aP2 similar to troglitazone. Furthermore, they increased PPARγ2 mRNA levels, different from troglitazone.

The CCAAT/enhancer-binding protein (C/EBP) family plays an important role in growth and differentiation in many tissues and cell types. Studies in adipogenic cell lines have shown that hormonal induction of differentiation is rapidly followed by the expression of C/EBPβ and δ. Levels of these proteins peak within several days and then begin to drift downward, coinciding with a rise in C/EBPα and PPARγ. C/EBPα and PPARγ induce changes in gene expression characteristic of mature adipocytes. Although the relation between C/EBPα and PPARγ is complicated, there is a cross-regulation between C/EBPα and PPARγ during adipogenesis and the major role of C/EBPα is centered on maintaining the expression of PPARγ and promoting full insulin sensitivity.[23–25]

In the present study, troglitazone significantly increased C/EBPα and β mRNA on day 8, but have a weak effect on C/EBPδ mRNA; the ratios of C/EBPα/β-actin, C/EBPβ/β-actin, and C/EBPδ/β-actin were 1.32 ± 0.10 (3 μM, p <0.05) and 2.30 ± 0.02 (10 μM, p <0.01), 1.66 ± 0.01 (3 μM, p <0.01) and 2.10 ± 0.10 (10 μM, p <0.01), and 1.01 ± 0.03 (3 μM) and 1.59 ± 0.15 (p <0.01), respectively. Compound **30** also increased C/EBPα and β mRNA levels, and **31** increased the mRNA expression of all three C/EBPs (Table 4). However, troglitazone (3 μM) did not increase PPARγ mRNA levels in consistent with our previous reports,[16–18] though its effect on the expression of C/EBPs mRNA was similar to that of **30**. Therefore, effects on C/EBPs levels at the initial differentiated stage and other transcription factors and co-activators[26,27] that induce PPARγ expression should be examined.

2.5. Agonistic activity for PPARγ

Since the profile of expression was different from that of troglitazone, a PPARγ agonist, we presumed that the mechanism of action of **30** and **31** was different from that of PPARγ agonists. Therefore, the agonistic activity of **30** and **31** was examined using a nuclear

Table 3
Effects of 3,4′,7-trimethylkaempferol (**26**), tetramethylkaempferol (**30**), and pentamethylquercetin (**31**) on 2-deoxyglucose (2-DG) uptake in 3T3-L1 cells

Concn (μM)	2-DG uptake (% of control)				
	3,4′,7-Trimethylkaempferol (**26**)	Tetramethylkaempferol (**30**)	Pentamethylquercetin (**31**)	Kaempferol (**19**)	Troglitazone
A: Chronic treatment with test compounds					
0 (DMSO	100.0 ± 4.0	100.0 ± 2.7	100.0 ± 4.1*		100.0 ± 4.0
1	108.5 ± 6.9	146.5 ± 9.2**	129.9 ± 3.3**		—
3	116.2 ± 3.1*	176.6 ± 8.0**	162.2 ± 2.3**		202.5 ± 3.5**
10	118.9 ± 4.9*	163.1 ± 5.6**	152.9 ± 5.2**		—
30	113.6 ± 3.9	135.8 ± 2.5**	127.3 ± 2.3**		—
B: Acute treatment with test compounds					
0 (DMSO)	100.0 ± 2.8	100.0 ± 1.8	100.0 ± 6.9*	100.0 ± 1.7	100.0 ± 7.2
3	72.2 ± 6.3**	82.7 ± 5.2**	80.1 ± 4.2*	63.0 ± 4.1**	96.6 ± 2.9
10	69.8 ± 5.0**	62.7 ± 3.5**	65.5 ± 8.1**	30.3 ± 2.3**	—
30	60.1 ± 2.2**	46.0 ± 0.9**	35.9 ± 0.6**	12.7 ± 2.2**	—

Refer to Section 4.5 for the experimental methods. Briefly, after the differentiation, the medium was replaced with the maintenance medium. On day 8, 3T3-L1 cells were incubated in medium without serum for 4 h, and then washed with PBS. After 20 min of incubation in PBS containing insulin (100 nM) (buffer A) with or without the test compound, 2-deoxy-D-(2,6-^{3}H)-glucose was added to the wells. After 10 min, the cells were washed with ice-cold PBS to terminate the reaction, and the radioactivity of the cells was measured.
A: The test compound dissolved in DMSO was added to the differentiation and maintenance media and buffer A.
B: The test compound dissolved in DMSO was added to buffer A.
Values represent the mean ± SEM (n = 4). Significantly different from the control group, *p <0.05, **p <0.01.

Table 4
Effects of tetramethylkaempferol (**30**) and pentamethylquercetin (**31**), and troglitazone on the gene expression of adiponectin, PPARγ2, GLUT4, aP2, C/EBPα, C/EBPβ, and C/EBPδ

	Ratio (target gene/β-actin mRNA)				
	Tetramethylkaempferol (**30**), Concn (μM)				Troglitazone
	0	3	10	30	3
Adiponectin	1.00 ± 0.08	1.81 ± 0.05*	2.05 ± 0.09**	2.90 ± 0.41**	3.60 ± 0.40**
PPARγ2	1.00 ± 0.07	1.67 ± 0.06**	1.81 ± 0.06**	2.11 ± 0.12**	1.15 ± 0.02
GLUT4	1.00 ± 0.03	2.38 ± 0.07**	2.54 ± 0.19**	2.61 ± 0.24**	4.04 ± 0.27**
aP2	1.00 ± 0.10	1.53 ± 0.05	2.02 ± 0.09*	2.24 ± 0.07**	3.17 ± 0.66**
C/EBPα	1.00 ± 0.04	1.88 ± 0.12**	2.08 ± 0.05**	1.82 ± 0.10**	1.32 ± 0.10*
C/EBPβ	1.00 ± 0.05	1.34 ± 0.12	1.89 ± 0.07**	3.14 ± 0.13**	1.66 ± 0.01**
C/EBPδ	1.00 ± 0.02	0.89 ± 0.07	1.23 ± 0.06	0.77 ± 0.05	1.01 ± 0.03
	Pentamethylquercetin (**31**), Concn (μM)				
	0	3	10	30	
Adiponectin	1.00 ± 0.04	1.67 ± 0.14**	1.91 ± 0.07**	2.59 ± 0.07**	
PPARγ2	1.00 ± 0.05	1.84 ± 0.06**	1.99 ± 0.07**	2.42 ± 0.18**	
GLUT4	1.00 ± 0.09	2.01 ± 0.07**	2.25 ± 0.08**	3.62 ± 0.21**	
aP2	1.00 ± 0.11	1.34 ± 0.03	1.59 ± 0.05**	2.10 ± 0.14**	
C/EBPα	1.00 ± 0.08	1.79 ± 0.09**	1.50 ± 0.10**	1.38 ± 0.02*	
C/EBPβ	1.00 ± 0.11	1.73 ± 0.11**	3.01 ± 0.08**	3.82 ± 0.23**	
C/EBPδ	1.00 ± 0.04	1.54 ± 0.19	1.93 ± 0.15**	2.34 ± 0.20**	

Refer to Section 4.6 for the experimental methods. Briefly, after the differentiation, the medium was replaced with the maintenance medium. On day 8, total RNA was extracted and reverse transcribed to cDNA. Then a real-time PCR was carried out. The abundance of each gene product was calculated by relative quantification, with values for the target genes normalized to β-actin mRNA. The test compound dissolved in DMSO was added to the differentiation and maintenance media.
Values represent the mean ± SEM (n = 3). Significantly different from the control group,
* p <0.05.
** p <0.01.

Table 5
Primers sequences for mouse adiponectin, PPARγ2, GLUT4, aP2, C/EBPs, and β-actin

Adiponectin	Sense	5′-AAGGACAAGGCCGTTCTCT-3′
	Antisense	5′-TATGGGTAGTTGCAGTCAGTTGG-3′
PPARγ2	Sense	5′-GGTGAAACTCTGGGAGATTC-3′
	Antisense	5′-CAACCATTGGGTCAGCTCTTG-3′
GLUT4	Sense	5′-CCTGAGAGCCCCAGATACCTCTAC-3′
	Antisense	5′-GTCGTCCAGCTCGTTCTACTAAG-3′
aP2	Sense	5′-AACACCGAGATTCCTTCAA-3′
	Antisense	5′-TCACGCCTTTCATAACACAT-3′
C/EBPα	Sense	5′-GCGGGCAAAGCCAAGAA-3′
	Antisense	5′-GCGTTCCCGCCGTACC-3′
C/EBPβ	Sense	5′-AAGAGCCGCGACAAGGC-3′
	Antisense	5′-GTCAGCTCCAGCACCTTGTG-3′
C/EBPδ	Sense	5′-TGCCCACCCTAGAGCTGTG-3′
	Antisense	5′-CGCTTTGTGGTTGCTGTTGA-3′
β-Actin	Sense	5′-ATGGGTCAGAAGGACTCCTACG-3′
	Antisense	5′-AGTGGTACGACCAGAGGCATAC-3′

Thermal cycling conditions for the PCR were 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s.

receptor cofactor assay system (EnBio RCAS for PPARγ, EnBioTec Laboratories). The EC_{50} of troglitazone was 1.0 μM in this system, but the compounds showed less activity (Fig. 3). These findings suggest that the mechanism of action by **30** and **31** is different from that by PPARγ agonists, and an enhancement of C/EBPs expression is involved in the PPARγ agonist-like effects of **30** and **31**.

3. Conclusion

In conclusion, several methoxyflavonols [3,4′,7-trimethylkaempferol (**26**), tetramethylkaempferol (**30**), and pentamethylquercetin (**31**)] promoted the adipogenesis of 3T3-L1 cells. Among them, **30** and **31** were most active and significantly increased the amount of adiponectin released into the medium and the uptake of 2-deoxyglucose into the cells. Compounds **30** and **31** also increased mRNA levels of adiponectin, PPARγ2, and GLUT4, but neither compound acted as a PPARγ agonist, different from troglitazone. These findings suggest that methoxyflavonols such as **30** and **31** are promising seed compounds for the development of antidiabetic agents, although their molecular targets need to be clarified.

4. Materials and methods

4.1. Chemicals and reagents

Natural flavonoids were isolated from medicinal herbs and identified as described in our previous papers.[5,6,28–30] Compounds **1**, **17**, **34**, and **44** were obtained from Funakoshi Co., Ltd. (Tokyo, Japan) and/or Wako Pure Chemical Co., Ltd. (Osaka, Japan).

Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma–Aldrich (St. Lois, MO). Penicillin and streptomycin were from Life Technologies Japan Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was from PAA Laboratories GmbH (Pasching, Austria). Quantikine Mouse Adiponectin/Acrp 30 Immunoassay was from R&D Systems, Inc. (Minneapols, MN). RNeasy™ Mini Kit was from

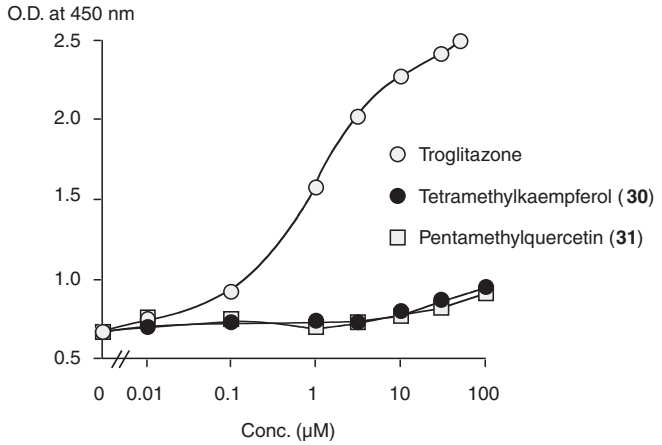


Figure 3. Agonistic activity of tetramethylkaempferol (**30**) and pentamethylquercetin (**31**), and troglitazone for PPARγ determined using a nuclear receptor cofactor assay system. Refer to Section 4.7 for the experimental methods. The experiment was done in duplicate.

Qiagen Inc. (Valencia, CA). ReverTra Ace® qPCR RT Kit and THUNDERBIRD™ SYBR® qPCR mix Kit were from Toyobo Co., Ltd (Osaka, Japan). 2-Deoxy-D-(2,6-^{3}H)-glucose (8.9 GBq/mg) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). EnBio RCAS for PPARγ was from EnBioTec Laboratories, Co., Ltd. (Tokyo, Japan). Triglyceride E-test Wako, 2-deoxy-D-glucose, cytochalasin B, and the other chemicals were from Wako Pure Chemical Co., Ltd.

The 48-well multiplates (Sumilon Cell Tight C-1), and the 6-well and 96-well multiplates (Sumilon) were purchased from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan).

4.2. Cell culture

Murine 3T3-L1 cells (Cell No. IFO50416) were obtained from Health Science Research Resources Bank (Osaka, Japan), and cultured in Dulbecco's modified Eagle's medium (DMEM) containing low glucose (1000 mg/L) supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) until confluent.

4.3. Adipogenesis

3T3-L1 cells (5.0×10^4 cells/150 μL/well) were seeded into a 48-well multiplate. After 24 h, differentiation was induced by changing the medium to a differentiation medium [DMEM (high glucose: 4500 mg/L) supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 5 μg/mL insulin]. After 3 days, the differentiation medium was replaced with a maintenance medium [DMEM (high glucose) supplemented with 10% FBS and 5 μg/mL insulin]. After 2 days (on day 6), the medium was exchanged with fresh medium, and the cells were continuously cultured for 2 days. On day 8, the medium was removed and H_2O (200 μL/well) was added to each well, and then the cells were sonicated. The TG level in the sonicate was determined with a commercial kit (Triglyceride E-test Wako). The test compound dissolved in dimethylsulfoxide (DMSO) was added to the differentiation and maintenance media (final DMSO concn, 0.1%). Troglitazone was used as a reference compound.

4.4. Adiponectin release

3T3-L1 cells (5.0×10^4 cells/well) in DMEM supplemented with 10% FBS were seeded into a 48-well multiplate. After the initial differentiation, the medium was replaced with the maintenance medium described above. After 4 days (on day 8), adiponectin concentrations in the medium were measured using an ELISA kit (R&D systems) according to the manufacturer's instructions. The test compound dissolved in DMSO was added to the differentiation and maintenance media (final DMSO concn, 0.1%).

4.5. Uptake of 2-deoxyglucose

3T3-L1 cells (5.0×10^4 cells/well) in DMEM supplemented with 10% FBS were seeded into a 48-well multiplate. After the initial differentiation, the medium was replaced with the maintenance medium described above. After 4 days (on day 8), the cells were incubated in medium without serum for 4 h, and then washed with PBS. After 20 min of incubation in PBS containing insulin (100 nM) (buffer A) with or without the test compound, 2-deoxy-D-glucose (final concn, 0.1 mM) and 2-deoxy-D-(2,6-^{3}H)-glucose (final concn, 1 μCi/mL) were added to the wells. After 10 min, the cells were washed with ice-cold PBS to terminate the reaction, and then a 1 M NaOH solution (200 μL/well) was added to each well and incubation was continued for 2 h at 37 °C to dissolve the cells. After neutralization with 1 M HCl, an aliquot was transferred to a vial and the radioactivity was measured using a liquid scintillation counter (Beckman LS6500). Nonspecific uptake was determined in the presence of 20 μM cytochalasin B and was subtracted from the total value. The cells were treated with the test compound as follows: (A) the test compound dissolved in DMSO was added to the differentiation and maintenance media and buffer A (final DMSO concn, 0.1%), and (B) the test compound dissolved in DMSO was added to buffer A (final DMSO concn, 0.1%).

4.6. Gene expression

3T3-L1 cells (1.0×10^6 cells/2 mL/well) in DMEM supplemented with 10% FBS were seeded into a 6-well multiplate. After the initial differentiation, the medium was replaced with the maintenance medium described above. After 4 days (on day 8), total RNA was extracted using an RNeasy™ Mini Kit (Qiagen) according to the manufacturer's instructions. The total RNA was reverse transcribed to cDNA using a ReverTra Ace® qPCR RT Kit (Toyobo). Then a real-time PCR was carried out on a MiniOpticon real-time machine (Bio-Rad) using a THUNDERBIRD™ SYBR® qPCR mix Kit (Toyobo). The abundance of each gene product was calculated by relative quantification, with values for the target genes normalized to β-actin mRNA, and the mean value of each control (vehicle) was calculated to be 1.00. Thermal cycling conditions for the PCR were 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, then a melting curve analysis from 65 to 95 °C, every 0.2 °C. The primer pairs for target genes (adiponectin,[31] PPARγ2,[32] GLUT4,[33] aP2,[32] C/EBPsα, β, and d,[34] and β-actin[35]) were purchased from Invitrogen (Table 5). The test compound dissolved in DMSO was added to the differentiation and maintenance media (final DMSO concn, 0.1%).

4.7. Agonistic activity for PPARγ

Agonistic activity for PPARγ was examined using a nuclear receptor cofactor assay system (EnBio RCAS for PPARγ, EnBioTec Laboratories) according to the manufacturer's instructions. This system is a cell-free assay system using nuclear receptors and cofactors to screen chemicals. The change in absorbance (450 nm) caused by troglitazone at 50 μM was calculated as 100% and the EC_{50} value was determined graphically. The experiment was done in duplicate.

4.8. Statistical analyses

Values are expressed as the mean ± SEM. A one-way analysis of variance followed by Dunnett's test was used for statistical analyses. Probability (p) values of less than 0.05 were considered to be significant.

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References and notes

1. Henness, S. *Curr. Opin. Endocrinol. Diabetes Obs.* **2007**, *14*, 166–169.
2. Knekt, P.; Kumpulainen, J.; Järvinen, R.; Rissanen, H.; Heliövaara, M.; Reunanen, A.; Hakulinen, T.; Aromaa, A. *Am. Journal. Clin. Nutr.* **2002**, *76*, 560–568.
3. Saito, T.; Abe, D.; Sekiya, K. *Biochem. Biophys. Res. Commun.* **2007**, *357*, 371–376.
4. Sharma, B.; Balomajumder, C.; Roy, P. *Food Chem. Toxicol.* **2008**, *46*, 2376–2383.
5. Matsuda, H.; Morikawa, T.; Toguchida, I.; Yoshikawa, M. *Chem. Pharm. Bull.* **2002**, *50*, 788–795.
6. Matsuda, H.; Wang, T.; Managai, H.; Yoshikawa, M. *Bioorg. Med. Chem.* **2003**, *11*, 5317–5323.
7. Harmon, A. W.; Patel, Y. M. *Biochem. Biophys. Res. Commun.* **2003**, *305*, 229–234.
8. Nomura, M.; Takahashi, T.; Nagata, N.; Tsutsumi, K.; Kobayashi, S.; Akiba, T.; Yokogawa, K.; Moritani, S.; Miyamoto, K. *Biol. Pharm. Bull.* **2008**, *31*, 1403–1409.

9. Iwaki, M.; Matsuda, M.; Maeda, N.; Funahashi, T.; Matsuzawa, Y.; Makishima, M.; Shimomura, I. *Diabetes* **2003**, *52*, 1655–1663.
10. Kadowaki, T.; Yamauchi, T. *Endocr. Rev.* **2005**, *26*, 439–451.
11. Parker, J. C. *Adv. Drug Deliv. Rev.* **2002**, *54*, 1173–1197.
12. Tsuchida, A.; Yamauchi, T.; Kadowaki, T. *Journal. Pharmacol. Sci.* **2005**, *97*, 164–170.
13. Tafuri, S. R. *Endocrinology* **1996**, *137*, 4706–4712.
14. Han, K. L.; Jung, M. H.; Sohn, J. H.; Hwang, J.-K. *Biol. Pharm. Bull.* **2006**, *29*, 110–113.
15. Shang, W.; Yang, Y.; Jiang, B.; Jin, H.; Zhou, L.; Liu, S.; Chen, M. *Life Sci.* **2007**, *80*, 618–625.
16. Zhang, H.; Matsuda, H.; Kumahara, A.; Ito, Y.; Nakamura, S.; Yoshikawa, M. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4972–4976.
17. Zhang, H.; Matsuda, H.; Nakamura, S.; Yoshikawa, M. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3272–3277.
18. Zhang, H.; Matsuda, H.; Yamashita, C.; Nakamura, S.; Yoshikawa, M. *Eur. Journal. Pharmacol.* **2009**, *606*, 255–261.
19. Go, M. L.; Wu, X.; Liu, X. L. *Curr. Med. Chem.* **2005**, *12*, 481–499.
20. López-Lázaro, M. *Mini-Rev. Med. Chem.* **2009**, *9*, 31–59.
21. Fresco, P.; Borges, F.; Marques, M. P.; Diniz, C. *Curr. Pharm. Des.* **2010**, *16*, 114–134.
22. Fang, X. K.; Gao, J.; Zhu, D. N. *Life Sci.* **2008**, *82*, 615–622.
23. Wu, Z.; Rosen, E. D.; Brun, R.; Hauser, S.; Adelmant, G.; Troy, A. E.; McKeon, C.; Darington, G. J.; Spiegelman, B. M. *Mol. Cell* **1999**, *3*, 151–158.
24. Porse, B. T.; Pederson, T. A.; Xu, X.; Lindberg, B.; Wewer, U. M.; Friis-Hansen, L.; Nerlov, C. *Cell* **2001**, *107*, 247–258.
25. Rosen, E. D.; Hsu, C. H.; Wang, X.; Sakai, S.; Freeman, M. W.; Gonzalez, F. J.; Spiegelman, B. M. *Genes Dev.* **2002**, *16*, 22–26.
26. Mori, T.; Sakaue, H.; Iguchi, H.; Gomi, H.; Okada, Y.; Takashima, Y.; Nakamura, K.; Yamauchi, T.; Kubota, N.; Kadowaki, T.; Matsuki, Y.; Ogawa, W.; Hiramatsu, R.; Kasuga, M. *Journal. Biol. Chem.* **2005**, *280*, 12867–12875.
27. Rosen, E. D.; MacDougald, O. A. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 885–896.
28. Yoshikawa, M.; Shimada, H.; Shimoda, H.; Murakami, N.; Yamahara, J.; Mastuda, H. *Chem. Pharm. Bull.* **1996**, *44*, 2086–2091.
29. Xu, F.; Matsuda, H.; Hata, H.; Sugawara, K.; Nakamura, S.; Yoshikawa, M. *Chem. Pharm. Bull.* **2009**, *57*, 1089–1095.
30. Sae-wong, C.; Matsuda, H.; Tewtrakul, S.; Tansakul, P.; Nakamura, S.; Nomura, Y.; Yoshikawa, M. *J. Ethnopharmacol*., in press (JEP-6553).
31. Aoyagi, T.; Shimba, S.; Tezuka, M. *Journal. Health Sci.* **2005**, *51*, 21–32.
32. Kudo, M.; Sugawara, A.; Uruno, A.; Takeuchi, K.; Ito, S. *Endocrinology* **2004**, *145*, 4948–4956.
33. McClain, D. A.; Hazel, M.; Parker, G.; Cooksey, R. C. *Am. Journal. Physiol. Endocrinol. Metab.* **2005**, *288*, E973–E979.
34. Waki, H.; Park, K-W.; Mitro, N.; Pei, L.; Damoiseaux, R.; Wilpitz, D. C.; Reue, K.; Saez, E.; Tontonoz, P. *Cell Metab.* **2007**, *5*, 357–370 (Supplementary data).
35. Ajuwon, K. M.; Spurlock, M. E. *Am. Journal. Physiol. Regul. Integr. Compd. Physiol.* **2005**, *288*, R1220–R1225.