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Mycophenolic acid as a latent agonist of PPARγ

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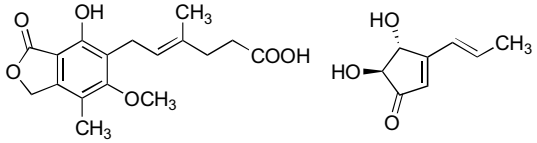
Abstract—Mycophenolic acid (MPA), known as an inhibitor of inosine monophosphate dehydrogenase (IMPDH), was found to inhibit the differentiation of 3T3-L1 pre-adipocytes into mature adipocytes. Although the effect of MPA was attributed to inhibition of IMPDH, we uncovered a hidden biological property of MPA as an agonist of peroxisome proliferator activated receptor γ (PPARγ).

Obesity, an excessive accumulation of adipose tissue, is a common disorder,[1] and white adipose tissue (WAT) mass is a reflection of the number of adipocytes and their volume.[2] Adipocyte differentiation from pre-adipocytes is therefore one of the major causes of the development of obesity. The model cell line, 3T3-L1 cells, can be induced to differentiate into mature adipocytes in cell culture.[3] Lovastatin,[4] ritonavir, nelfinavir,[5] 6-ethoxyzolamide,[6] and prostaglandin $F_{2\alpha}$, fluprostenol,[7] wortmannin[8] have been reported as inhibitors of adipocyte differentiation. Recently we reported several germacranolides from *Calea urticifolia* as inhibitors of 3T3-L1 pre-adipocyte differentiation.[9] In this study, we report new biological properties of mycophenolic acid (MPA) (**1**) as a latent agonist of peroxisome proliferator activated receptor γ (PPARγ) and a direct evidence of molecular interaction between **1** and PPARγ.

During the course of our screening program for the modulator of 3T3-L1 cell differentiation, we found that two natural compounds, MPA (**1**) isolated from a broth of *Penicillium* sp., BAUA 2775, and terrein (**2**)[10] from a broth of an unidentified fungus BAUA 4031 (donated by Kaken Pharmaceutical Co., Ltd), block insulin-induced differentiation of 3T3-L1 pre-adipocytes into mature adipocytes (Fig. 1).

Terrein (**2**) showed weak inhibitory activity with minimal inhibitory concentration at 81 μM, which was observed by diminishing oil-red O stained cells under a microscope. However, **1** showed potent inhibitory activity. The minimal effective concentration of a highly purified sample was 0.2 μM.

To clarify structure–activity relationship among the derivatives of **1**, we prepared 17 derivatives of **1**. Scheme 1 shows the synthesis of some important derivatives, of which C-7-OH, C-14 carboxylic acid, and C-5-OMe were modified to several other functional groups.



Mycophenolic acid (**1**) Terrein (**2**)

Figure 1. Structures of inhibitors of 3T3-L1 pre-adipocyte cell differentiation, mycophenolic acid (MPA) (**1**) and terrein (**2**).

Keywords: Mycophenolic acid; IMPDH; PPARγ; 3T3-L1; Inhibitor; SPR.
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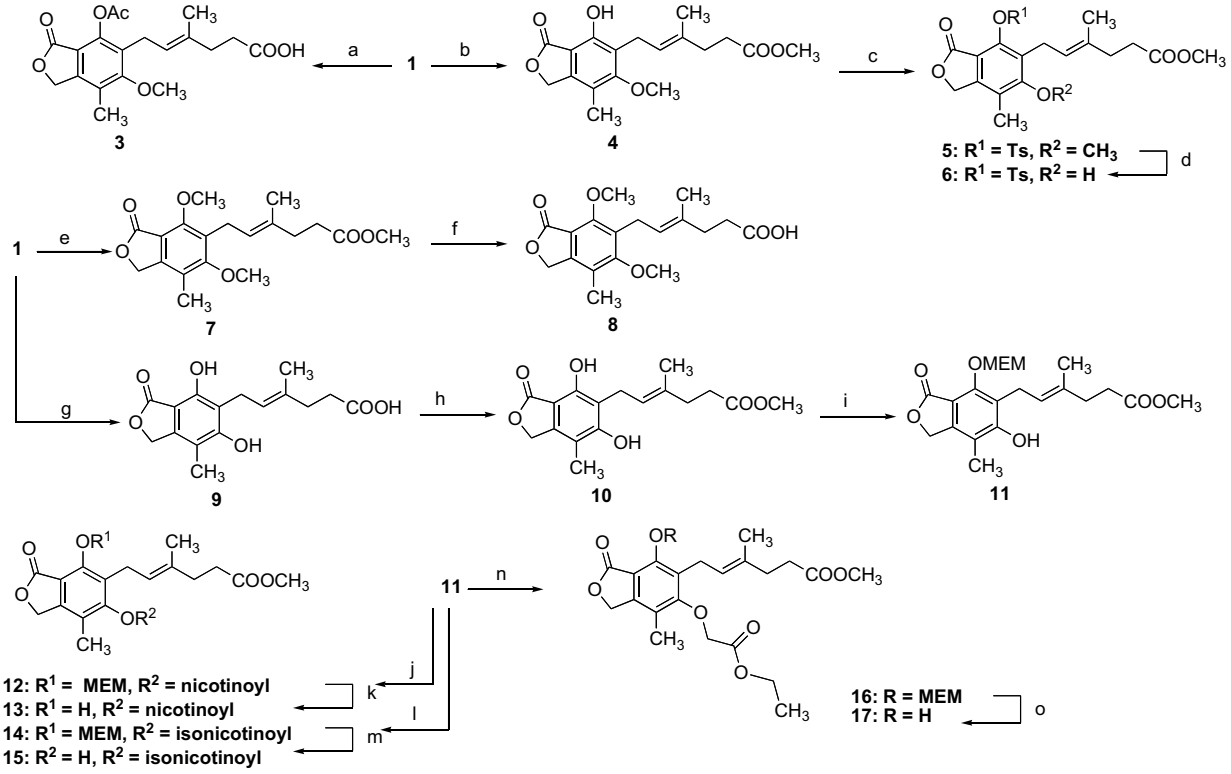

Scheme 1. Reagents and conditions: (a) Ac_2O, DMAP, Py, rt, 62%; (b) TsOH, MeOH, rt, 9 h, 78%; (c) TsCl, Et_3N, CH_2Cl_2, rt, 4 h, 96%; (d) LiI, Py/collidine (1:5), 150 °C, 15 h, 23%; (e) $TMSCHN_2$, MeOH, rt, 12 h, 90%; (f) LiOH, H_2O/acetone (1:4), 2 h, 33%; (g) LiI, Py/collidine (1:5), 120 °C, 18 h, 74%; (h) TsOH, MeOH, rt, 9 h, 78%; (i) MEMCl, NaH, THF, 0 °C–rt, 9 h, 44%; (j) nicotinoyl chloride hydrochloride, py, rt, 4 h, 56%; (k) TFA, CH_2Cl_2, rt, 12 h, 83%; (l) isonicotinoyl chloride hydrochloride, DMAP, Py, rt, 4 h, 67%; (m) TFA, CH_2Cl_2, rt, 12 h, 81%; (n) ethylbromoacetate, NaH, THF, 0 °C–rt, 95%; (o) TFA, CH_2Cl_2, rt, 12 h, 61%.

Among the derivatives, only two compounds, 7-*O*-acetyl MPA (**3**) and methyl ester derivative (**4**), showed the inhibitory activity of 3T3-L1 pre-adipocyte cell differentiation at 2 μM, while **1** showed the same activity at 0.2 μM. Compounds **5**–**17** did not inhibit the adipocyte differentiation of 3T3-L1 cells at 2 μM. 7-*O*-Acetyl MPA (**3**) was prepared from **1** with Ac_2O in the presence of pyridine and DMAP, and methyl ester derivative of MPA (**4**) was prepared from **1** with *p*-toluenesulfonic acid in methanol. 7-*O*-Methyl MPA (**8**) was prepared via trimethyl derivative (**7**), which was derived from **1** by using $TMSCHN_2$. Replacements of 5-OMe functional group to 5-*O*-nicotinoyl (**13**), 5-*O*-isonicotinoyl (**15**) functional moieties were achieved by demethylation of **1** using modified protocol of a previously reported method,[11] followed by conversion of carboxylic acid into methyl ester, regioselective protection of C-7-OH with MEMCl, esterification with nicotinoyl chloride, or isonicotinoyl chloride and deprotection of MEM group. 5-*O*-Ethoxycarbonylmethyl derivative (**17**) was synthesized using similar protocol (Scheme 1).

To determine quantitative inhibitory activities of **1**, **3**, **4**, **8**, **11**, **15**, and **17**, we measured the inhibitory activity against [1-^{14}C]sodium acetate uptake into 3T3-L1 cells, which is one of the markers of lipid biosynthesis.[3] Although, the inhibitory activities on the sodium acetate uptake are not wholly parallel to the inhibitory activities of 3T3-L1 cell differentiation, **1** and its analogs **3** and **4** clearly inhibited at 2 μM, whereas 5-*O*-isonicotinoyl derivative (**15**) and 5-*O*-ethoxycarbonylmethyl derivative (**17**) did not inhibit at the same concentration, which could be used for further biological investigation as inactive derivatives. Conversion of the phenolic hydroxy group at C-7 position into 7-*O* ether functional group resulted in a decrease in the inhibitory activity, thus 7-OMe MPA (**8**) and methyl ester derivative of 7-*O*-MEM MPA (**11**) showed weak inhibitory activity (14% and 10%, respectively) of the [1-^{14}C]sodium acetate uptake at 2 μM (Table 1).

Next we focused our attention on the molecular target of **1**. Differentiation of HL60 cells by **1** is associated with substantial decreases in both the guanylate and adenylate pools and appeared to be dependent on the state of depletion of intracellular GTP.[12] Simultaneous addition of guanosine or guanine to **1**-treated cells restored the GTP pool and prevented differentiation from occurring.

Table 1. Inhibitory activities of [1-^{14}C]sodium acetate uptake into 3T3-L1 cells by MPA (**1**) and its analogs

Compound	Inhibitory activity (%) at 2 μM
1	74
3	86
4	67
8	14
11	10
15	3
17	0

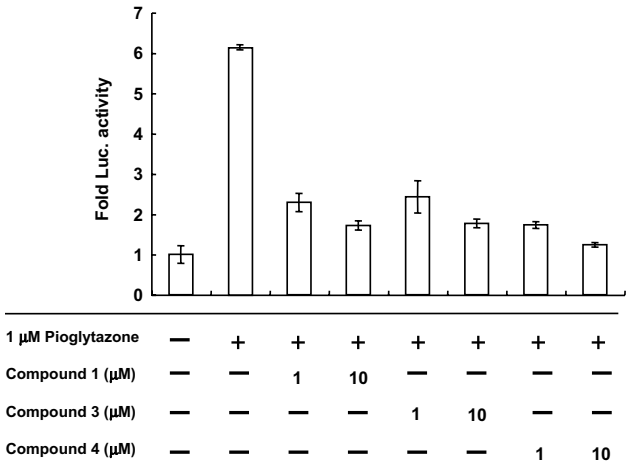

Figure 2. Partial antagonistic activities of MPA (**1**) and its derivatives (**3** and **4**) against pioglitazone (Pio) on PPARγ activation. U2OS cells were transfected with expression plasmids for nuclear receptor, together with the corresponding reporter plasmids and pCMV-β-galactosidase as internal control. Values are means of triplicate cultures.

Although the effect of **1** on 3T3-L1 cells is inhibitive and not inductive, it was considered that inhibition of purine and pyrimidine nucleotide biosynthesis is also important in this case. To confirm this idea, we achieved similar experimental tests for **1**-treated 3T3-L1 cells. First of all, we improved the culture conditions to obtain stable results concerning the differentiation of 3T3-L1 cells in our laboratory in Sapporo. Second, we actualized quantification of differentiation of 3T3-L1 cells based on quantitative determinations of triglyceride and DNA.[13] Under these cultural conditions, it was finally suggested that the depletion of guanine ribonucleotide is the obligatory step toward the inhibition of differentiation in **1**-treated 3T3-L1 cells, and hidden biological properties of **1** toward 3T3-L1 were uncovered as described later. Ribavirin[14] and oxanosine, the putative precursor of oxanosine-5′-phosphate,[15] which are known as inhibitors of IMPDH, did not show the same effects as **1** in our experiments. Thus **1** could conceivably be a unique molecule among the class of IMPDH inhibitors.

Recently we found that 4-*O*-carboxymethyl-2-*O*-methylascochlorin and 4-*O*-isonicotinoyl-2-*O*-methylascochlorin are potent agonists of PPARγ.[16] From the standpoint of structural similarities between **1** and ascochlorin, we next estimated the effect on the peroxisome proliferator activated receptor γ (PPARγ), which was determined as a critical nuclear receptor on adipocyte differentiation. Transcriptional activity of PPARγ stimulated with pioglitazone was inhibited by **1**, **3**, or **4** even at the dose of 1 μM (Fig. 2). These observations suggest that **1** is a ligand of PPARγ.

To obtain direct evidence that **1** is a ligand of PPARγ, characterization of the affinity and rate of the molecular interaction between **1** and PPARγ were performed by surface plasmon resonance (SPR) method.[17] Molecular interaction between **1** and PPARγ was recorded on a Biacore X (Biacore, Sweden). A recombinant human PPARγ GST fusion protein was immobilized on a Sensor Chip CM5 with amine coupling using PBS buffer (pH 4.0). Excess reactive groups on the surface were deactivated with 1 M ethanolamine hydrochloride. The amount of immobilized PPARγ GST fusion protein was adjusted to 13,000RU. The same equivalent molar of GST protein (300RU) was captured on a Sensor Chip CM5 as a reference using a similar amine coupling method. A recombinant human PPARα GST fusion was immobilized by similar manner. The amount of immobilized protein was 15,600RU. The same equivalent of GST protein (390RU) was immobilized as a reference. We determined the analytical conditions such as concentration of DMSO, selection of buffer, and sample preparations using ciglitazone ($K_D = 1.91 \times 10^{-4}$ M, PBS-N buffer), which is known as an agonist of PPARγ. Analyte (**1**) dissolved in 5% DMSO in PBS-N buffer (67 mM Na_2HPO_4, 12.5 mM KH_2PO_4, 70 mM NaCl) was loaded on the Sensor Chip. The kinetics of the analyte–ligand interaction was determined by a nonlinear fitting method using BIAevaluation software (Biacore, Sweden). Using an indirect method for kinetic analysis, the dissociation constant (K_D) between **1** and PPARγ was determined to be 1.10×10^{-4} M ($R_{max} = 17.7$, $\chi^2 = 2.04 \times 10^{-4}$) as shown in Figure 3, whereas the

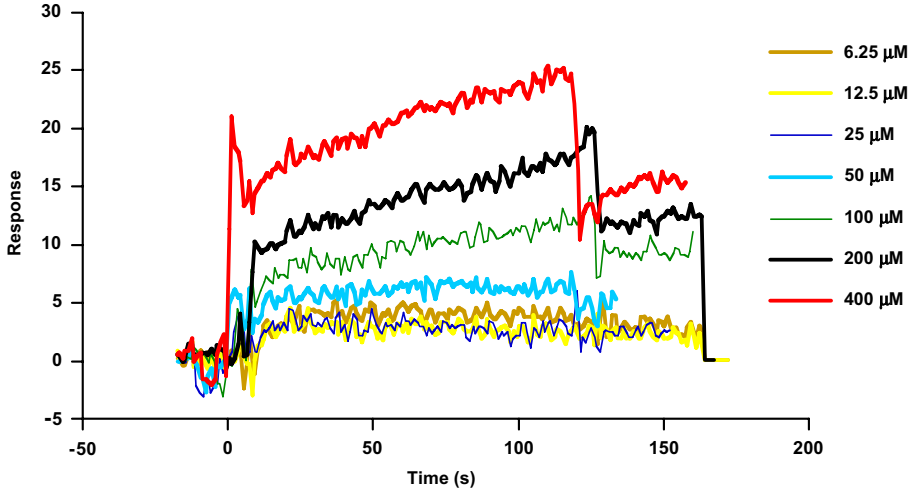

Figure 3. Sensorgram showing the interaction of **1** with PPARγ.

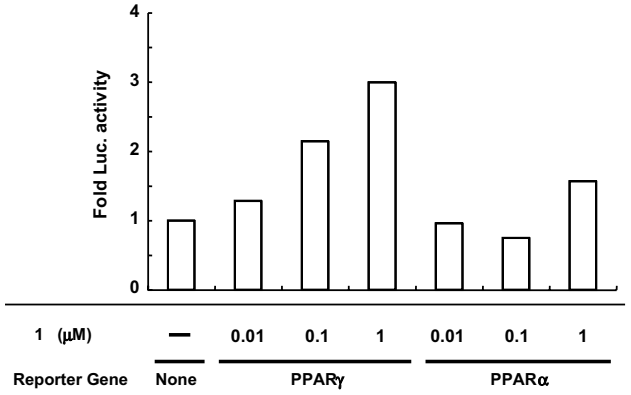

Figure 4. Agonistic activities for PPARγ and PPARα of MPA (**1**). U2OS cells were transfected with expression plasmids for nuclear receptors (PPARγ or PPARα), together with the corresponding reporter plasmids and pCMV-β-galactosidase as internal control.

dissociation constant between one of thiazolidinedione, troglitazone, and PPARγ was 6.15×10^{-6} M ($R_{max} = 12.7$, $\chi^2 = 0.00893$). The dissociation constant between **1** and PPARα could not be deduced, because of the adsorption of **1** to the reference cell. However, it was determined to be 5.08×10^{-4} M ($R_{max} = 25.2$, $\chi^2 = 0.131$) using PBS-P buffer (PBS-N + 0.005% Tween 20). The dissociation constant of troglitazone and PPARα was 5.22×10^{-5} M ($R_{max} = 42.2$, $\chi^2 = 0.192$) using PBS-N buffer.

The interaction between **1** and PPARγ was comparable to the dissociation constants of some of thiazolidinedione analogs.[18] These results showed the direct evidence of the molecular interaction between **1** and PPARγ.

To determine if **1** acts on PPARγ as an agonist in vivo, we conducted a reporter assay of **1** without pioglitazone. MPA (**1**) activated the transcriptional activity of PPARγ at the concentrations of 0.01–1 μM as shown in Figure 4. Moreover, **1** stimulated adipocyte differentiation of 3T3-L1 cells at the concentration of 2–4 μM in the presence of 50 μM of guanosine, which diminishes IMPDH activity of **1** (data not shown).

In conclusion, we succeeded in uncovering a molecular target of **1** except IMPDH. These findings will provide a new insight into the drug discovery utilizing **1**.

Acknowledgments

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