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Structure–activity relationships for inhibition of inosine monophosphate dehydrogenase and differentiation induction of K562 cells among the mycophenolic acid derivatives

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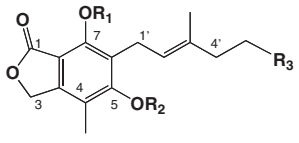
ABSTRACT

Inosine monophosphate dehydrogenases (IMPDHs) are the committed step in de novo guanine nucleotide biosynthesis. There are two separate, but very closely related IMPDH isoenzymes, termed type I and type II. IMPDHs are widely believed to be major targets for cancer and transplantation therapy. Mycophenolic acid (MPA) is a potent inhibitor of IMPDHs. Previously, we found that MPA acted as a latent agonist of this nuclear hormone receptor in U2OS cells, and 6′-hydroxamic acid derivatives of MPA inhibited tubulin-specific histone deacetylase[s] (HDAC[s]) in HeLa cells. Although MPA is a promising lead compound, structure–activity relationships (SARs) for inhibition of IMPDH, and the mechanism action of MPA derivatives have not well been understood. We therefore synthesized, evaluated MPA derivatives as IMPDH inhibitor in vitro and cellular level, and explored their biological function and mechanism in cultured cells. This paper exhibits that (i) functional groups at C-5, C-7, and C-6′ positions in MPA are important for inhibitory activity against IMPDH, (ii) it is difficult to improve specificity against IMPDH II by modification of 5-, 7-, and 6′-group, (iii) demethylation of 5-OMe results in increasing hydrophilicity, and lowering cell permeability, (iv) ester bonds of protective groups at C-7 and C-6′ positions are hydrolyzed to give MPA in cultures, (v) the effects of a tubulin-specific HDAC[s] inhibitor on proliferation and differentiation are weaker than its inhibitory activity against IMPDH. The present work may provide insight into the development of a new class of drug lead for treating cancer and transplantation.

1. Introduction

Mycophenolic acid (MPA, **1**, Fig. 1) is a potent uncompetitive inhibitor of human inosine monophosphate dehydrogenases (IMPDHs).[1–7] IMPDHs catalyzes the nicotinamide adenine dinucleotide (NAD)-dependent oxidation of inosine-5-monophosphate (IMP) to xanthosine-5-monophosphate (XMP), which is the committed step in de novo guanine nucleotide biosynthesis.[1,8] This reaction is particularly important to B and T lymphocytes, which are significantly dependent on the de novo pathway rather than the salvage pathway in purine biosynthesis. Thus the prodrug mycophenolate mofetil (MMF) has been used in organ and stem cell transplantation and in autoimmune diseases as a highly effective immunosuppressive agent.[1,9]

There are two separate, but very closely related IMPDH isoenzymes, termed type I and type II, that share 84% amino acid identity. In general, IMPDH I is expressed constitutively at low levels, while IMPDH II is amplified during proliferation, though several exceptions to this rule exist. Expression of IMPDHs, particularly the type II enzyme, is significantly up-regulated in many tumor cells including leukemia cells. IMPDHs are widely believed to be major targets for cancer as well as immunosuppressive chemotherapy.[1–5] MPA, which inhibits both type I and type II, can effectively



1: R_1= H, R_2= CH_3, R_3= COOH
2: R_1= H, R_2= H, R_3= COOH
3: R_1= $COCH_3$, R_2= CH_3, R_3= COOH
4: R_1= CH_3, R_2= CH_3, R_3=COOH
5: R_1= H, R_2= CH_3, R_3= $COOCH_3$
6: R_1= H, R_2= CH_3, R_3= CH_2OH
7: R_1= H, R_2= CH_3, R_3= CONHOH

Figure 1. Structures of mycophenolic acid and its derivatives.

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induce cell-cycle arrest at the late G1 phase in lymphocytes,[3] causing differentiation or apoptosis depending on the cell type.[1,3] Due to its properties, MPA has entered phase I clinical trials in advanced multiple myeloma patients. Furthermore, it is expected that transplantation therapy and chemotherapy could be improved with IMPDH II specific inhibitors.[1–5] However, there is no IMPDH II specific inhibitor despite several studies.[5,6]

We have previously found that MPA inhibited adipocyte differentiation of 3T3-L1 cells.[10] Although this effect was attributed to the inhibition of IMPDHs, another molecular target of MPA was found to be PPARγ, and MPA was a latent agonist of this nuclear hormone receptor. 7-*O*-Acetyl MPA (**3**) and mycophenolic methylester (**5**), which are MPA derivatives, could inhibit PPARγ activation similarly to MPA.[10] Hence, analyses of the action mechanism of MPA analogs are informative for development of new drugs.

Furthermore, we designed and synthesized the hydroxamic acid derivatives of MPA having effective metal-binding site, mycophenolic hydroxamic acid (MPHA, **7**), 7-*O*-acetyl MPHA, 7-*O*-lauroyl MPHA, and 7-*O*-methyl MPHA.[11] Although MPA did not inhibit histone deacetylases (HDACs), all these derivatives inhibited HDACs in nuclear extract with IC_{50} values of 1, 0.9, 0.5, and 0.9 μM, and HeLa cell proliferation with IC_{50} values of 2, 1.5, 1, and 50 μM, respectively.[5,11] HDACs catalyze the removal of acetyl groups from lysine residues of histones. HDACs are also involved in regulating the acetylation of a number of non-histone proteins, such as α-tubulin and the tumor suppressor p53.[11,12] To date, four classes of human HDACs with 18 members have been identified. These HDACs are subdivided into three individual classes based on structural and functional similarities. The class I isoforms (HDACs 1, 2, 3 and 8) and class II (HDACs 4, 5, 6, 7, 9, 10 and 11) are Zn-dependent enzymes, whereas class III HDACs [Sir1–Sir7 (sirtuins 1–7)] are NAD^+-dependent.[12] A number of HDACs inhibitors are currently undergoing phase I, II and III clinical trials as anticancer drugs, some of which have been characterized as selective HDAC inhibitors and some as pan-HDAC inhibitors.[12,13] Anti-cancer effect of HDACs inhibitors is based on a fundamentally different mechanism than that caused by inhibition of IMPDH. Our previous results suggested that substitution of 7- or 6′-group was better option for conversion of MPA to HDACs inhibitors.[11] Thus, conversion of MPA into dual inhibitor of IMPDH and HDAC would give rise to a beneficial drug. However, structure–activity relationship (SAR) between the derivatives at C-7 or C-6′ in MPA and IMPDH inhibition, which becomes the basis for development of such dual inhibitor, has not been understood well.

In this paper, we describe synthesis and evaluations of MPA derivatives at C-7 or C-6′ as IMPDH inhibitor in vitro and at the cellular level, and mechanism of action of these derivatives in cultured cells.

2. Materials and methods

2.1. Chemicals

Mycophenolic acid (MPA, **1**) was purchased from Sigma-Aldrich. Compounds **2**–**5**, and **7** were synthesized according to our previous procedure.[10,11] All chemicals were purchased in highest purity and were used without further purification. Thin layer and column chromatography (CC) were performed with Merck Silica Gel 60 F_{254} and Kanto Chemicals Co. (Japan) Silica Gel 60N (spherical, neutral), respectively. NMR spectra were recorded on a Bruker AMX-500 spectrometer. Chemical shifts are reported in ppm (δ-scale) using TMS as the internal standard. Coupling constants (*J*) are given in Hertz. MS spectra were accomplished on JMS-SX102A equipment using EI and FAB techniques. The NMR and MS spectra were obtained at the GC–MS & NMR Laboratory, Faculty of Agriculture, Hokkaido University, Sapporo, Japan.

2.1.1. 7-Hydroxy-6-[(*E*)-6-hydroxy-3-methylhex-2-enyl]-5-methoxy-4-methyl-3*H*-2-benzofuran-1-one (6)

A solution of **5** (1.25 g, 3.75 mmol) in THF (4 mL) was added dropwise to a stirred suspension of $LiAlH_4$ (142 mg, 3.75 mmol) in THF (40 mL) at 0 °C under argon. After 15 min, the reaction was quenched by adding 1 M HCl (20 mL) and partitioned between EtOAc. The aqueous layer was extracted three times with EtOAc, and the combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo to give crude product. The crude product was chromatographed on a silica gel column, eluting with *n*-hexane/EtOAc (2:1), to give the starting material (309 mg, 24.7%) and desired alcohol as a colorless solid (582 mg, 51%).

NMR δ_H ($CDCl_3$): 7.69 (br s, 1H), 5.25 (t, J = 5.6 Hz, 2H), 5.21 (s, 2H), 3.78 (s, 3H), 3.61 (t, J = 6.2 Hz, 2H), 3.40 (d, J = 6.9 Hz, 2H), 2.15 (s, 3H), 2.07 (t, J = 7.4 Hz, 2H), 1.81 (s, 3H), 1.67 (q, J = 6.9 Hz, 2H), 1.36 (br s, 1H). NMR δ_C ($CDCl_3$): 172.9, 163.6, 153.6, 143.9, 135.6, 122.2, 116.6, 106.3, 69.9, 62.7, 60.9, 36.0, 30.6, 22.5, 16.0, 11.5. HR-FD-MS m/z ($[M]^+$) Calcd for $C_{17}H_{22}O_5$: 306.1467. Found: 306.1474.

2.2. IMPDH activity assay

Two plasmids for expression of human IMPDH I and IMPDH II were kindly given from Dr. L. Hedstrom (Brandeis University, USA).[14–16] The plasmid was transformed into *Escherichia coli* strain BL21(DE3). Transformed cells were cultured for 17 h at 170 rpm in 50 ml of fresh 2 × YT broth (1.6% bacto tryptone, 1.0% bacto yeast extract, 0.5% NaCl, pH 7.0) containing 100 μg/ml ampicillin. After seed culture, cell culture broth was diluted 14 times into 2 × YT containing 100 μg/ml ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside to induce overexpression of IMPDH, and the cells were further cultured for 280 min at 170 rpm in 25 °C. All of the following procedures were carried out at 4 °C. The cells were collected by centrifugation and suspended in 70 ml of buffer A (30 mM Tris–HCl pH 8.0 [4 °C], 1 mM EGTA, 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM PMSF and 10 μg/ml leupeptin). Cells were lysed by sonication and the lysate was centrifuged at 9000 rpm for 10 min. Protein concentration in the resulting supernatants were measured by the modified Bradford method using BSA as a standard,[17] and diluted to 2 mg/ml with a buffer A. Diluted supernatant was subjected to fractionation by ammonium sulfate precipitation. The precipitate (25–40% saturation) was resuspended in 30 ml of buffer B (15 mM Tris–HCl pH 8.0 [4 °C], 0.5 mM EGTA, 0.5 mM dithiothreitol, and 0.5 mM $MgCl_2$), and dialyzed for 15 h against two liter of buffer B. The dialysate was cleared by filtration (0.20 μm of cellulose acetate; Millipore, Germany), and concentrated 10 times by ultrafiltration (Vivaspin 20, 10 kDa molecular mass cut-off; GE healthcare). And then, IMPDH fraction was mixed with an equal volume of glycerol and stored at −20 °C until use as a source of enzyme.

The source of enzyme was diluted with BSA solution to 5.75 mU/ml IMPDH activity and 1 mg/ml protein concentration. Eighty microliter of reaction mixture containing 37.5 mM NaH_2PO_4 [pH 7.4], 125 mM KCl, 0.625 mM IMP and 1.25 mM β-NAD^+ with or without the compound was preincubated for 15 min at 37 °C. The reaction was initiated with 20 μl of diluted enzyme solution. After 60 min at 37 °C, the reaction was stopped by adding 15 μl of 2.5 M TCA, and then the solution was centrifuged for 10 min at 13,000 rpm. Supernatants were cleared by filtration (0.45 μm of cellulose acetate; ADVANTEC, Japan) before HPLC analyses (Table 1).[18] One unit of the enzyme is defined as the amount of enzyme required to catalyze 1 μmol of substrate per min.

2.3. Cell culture and proliferation assay

Erythroleukemia cell line K562 was maintained in RPMI-1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum

Table 1
HPLC protocol for the separation and quantification of XMP in IMPDH reaction mixture

Column	Octadecyl silane (ODS), Mightysil RP-18 GP 250–4.6 (5 μm), (250 × 4.6 mm I.D.; Kanto Chemicals Co.)
Guard column	Octadecyl silane (ODS), Mightysil RP-18 GP 5–4.6 (5 μm)
Mobile phase	20 mM H_3PO_4, 3 mM $MgSO_4$, pH 6.4 (triethylamine [HPLC grade, Thermo Scientific])
Flow rate	1.2 ml/min
Injection volume	20 μl
Column temperature	27 °C
Detection	240 nm
Retention time (XMP)	6.5 min
Retention time (IMP)	9.6 min

Table 2
HPLC protocol for the analyses of MPA and compound **5** in extracts from K562 cell culture medium

Column	ODS, Mightysil RP-18 GP 250–4.6 (5 μm), (250 × 4.6 mm I.D.; Kanto Chemicals Co.)
Guard column	ODS, Mightysil RP-18 GP 5–4.6 (5 μm)
Mobile phase	60% methanol, 0.1% acetic acid
Flow rate	1.0 ml/min
Injection volume	20 μl
Column temperature	40 °C
Detection	305 nm
Retention time (MPA)	11.0 min
Retention time (compound **5**)	19.0 min

(FBS), 2 g/l sodium bicarbonate, 100 μg/ml streptomycin, and 20 U/ml penicillin G at 37 °C under 5% CO_2. Logarithmically growing K562 cells (0.5×10^5 cells/ml) were plated on 6-well plate (1×10^5 cells per well), and then the cells were treated with compound dissolved in DMSO at various concentrations for 72 h. Cellular proliferation was measured using Cell Counting Kit-8 (DOJINDO LABORATORIES, Japan) and trypan blue exclusion assay.

2.4. Induction of K562 cell differentiation

This assay is based on the catalytic action of hemoglobin on the oxidation of *o*-dianisidine by hydrogen peroxide.[19] Logarithmically growing K562 cells (0.5×10^5 cells/ml) were plated on 6-well plate (1×10^5 cells per well) in cultured medium and the compounds were added to various concentrations. And then, cells were incubated for 72 h and the numbers of viable cells were counted as described in the section of cell culture and proliferation assay. To detect hemoglobin synthesis, cells (1×10^5 cells) were suspended in 1 ml of cultured medium, and added 50 μl staining cocktail of 1:24 ratio of 30% H_2O_2 to filtrated *o*-dianisidine solution (2.63% *o*-dianisidine in 5.0% acetic acid), and further incubated for 20 min at 900 rpm at 37 °C with mixer (thermomixer comfort, Eppendorf). Stained cells were washed twice by centrifugation for 2 min at 3400 rpm using PBS (−), and then suspended in 250 μl of TE buffer (10 mM Tris–HCl pH 8.0 and 1 mM EDTA) containing 1% SDS. Cells were lysed well by sonication, and put into each well of a 96-well plate (200 μl per well). The absorbance at 450 nm of each lysate was measured as an index of the amount of hemoglobin. To correct the amount of hemoglobin, lysate was diluted forty times with TE buffer, and quantified double-stranded DNA (Genomic DNA) using PicoGreen dsDNA Quantitation Reagent (Molecular probes).

2.5. Conversion of compounds 3 and 5 into MPA in the growth medium of K562 cells

Five × 10^5 K562 cells were cultured in 10 ml of culture medium in 10 cm dishes. Ten microlitre of DMSO or DMSO plus 20 nmol of MPA, **3** or **5** was added, and the cells were incubated at 37 °C for 0–72 h. Non-adherent cells and incubated medium were separated by centrifugation at 1100 rpm for 10 min and then corrected cells were suspended in 1.5 ml acetone. To the suspension, 10 ml of culture medium was added, and acetone was evaporated in vacuo. The resultant aqueous solution was adjusted to pH 4.0 with 2 M HCl, followed by $CHCl_3$ extraction and evaporation to give crude extract. The extract was dissolved in 100 μl of methanol, and cleared by filtration through cellulose acetate (0.45 μm, ADVANTEC, Japan) before HPLC analyses (Table 2).

2.6. Mathematical analyses

IC_{50} values were calculated with KaleidaGraph 3.6J software (HULINKS, Inc., Japan). The data was fitted to the dose–response logistic curve (sigmoid curve) with nonlinear regression produced by using the Levenberg–Marquardt algorithm in the software.

For estimation of octanol–water distribution coefficients (Log *D*) at pH 7.5 and pK_a of functional group in compounds, SPARC v4.5 on line calculator was used.[20]

3. Results and discussion

In order to evaluate SAR of MPA, we synthesized compounds **2**–**7** (Fig. 1), in which the 5-OMe group of MPA was demethylated (**2**), the hydroxyl group at C-7 was substituted (**3** and **4**), and carboxylic acid of MPA was replaced (**5**–**7**). Compounds **2**–**6** were previously reported, of which studies were evaluated for antitumor activity, and compounds **2**–**6** could be put in the following order of their decreasing activity: MPA > **3** > **5** > **6** = **2** ≫ **4**.[21] However, inhibitory effects of these compounds on IMPDH activity were not measured.[21]

Table 3 represents inhibitory activity of a set of compounds, **1**–**7** against IMPDH I and IMPDH II (see Fig. S1, Supplementary data). None of these modifications have provided any better inhibitor than MPA. Compound **2** showed most potent inhibitory activity among the all derivatives tested. The 5-OMe group interacts with carboxylic acid of Asp274 in IMPDH II, and replacement of 5-OMe to 5-OH did not strongly disturb its interaction. It is known that the 7-OH group is important for inhibitory activity of MPA. Some attempts to protect this group or to replace it with the F, NH_2, CN groups resulted in the loss of activities. In our experiment, **3** and **4** did not show potent activity against IMPDH I and IMPDH II, too. On the other hand, compound **7** previously reported as a dual inhibitor did not lose inhibitory activity against IMPDH II (K_i = 30 nM) and was found to have a similar potency to MPA (K_i = 10 nM).[5] However, IC_{50} value of **7** (0.42 μM) against IMPDH II is 35-fold larger than that of MPA (0.012 μM) as shown in Table 3. Inhibitory activities of **5** and **6** against IMPDH II are 117- and 66-fold less potent than that of MPA, respectively. These results indicate that carboxylic acid at C-6′ is also important for the inhibitory activity of MPA, and none of these derivatives exhibited better selectivity than MPA.

Table 4 shows inhibition of cell proliferation by a series of compounds, **1**–**7** and their partition coefficients between octanol and water at pH 7.5. MPA exhibited most potent inhibitory activity against K562 cells. These compounds could be put in the following order of their decreasing activity: MPA > **3** = **5** > **7** > **6** > **2** > **4**. It is noteworthy that **2** showed the largest IC_{50} ratio (K562 IC_{50}/IMPDH II IC_{50}). Replacing the 5-OMe group by 5-OH resulted in 15-fold

Table 3
Inhibition of IMPDH activity by MPA and its derivatives

Compound	IC_{50}[a] (μM)		IC_{50} ratio[b]
	IMPDH I	IMPDH II	
1	0.019 ± 0.001	0.012 ± 0.001	1.6
2	0.15 ± 0.01	0.17 ± 0.01	0.88
3	4.1 ± 0.6	4.1 ± 0.6	1.0
4	67 ± 8	72 ± 9	0.93
5	1.5 ± 0.1	1.4 ± 0.2	1.1
6	0.77 ± 0.06	0.79 ± 0.11	0.97
7	0.35 ± 0.02	0.42 ± 0.09	0.83

[a] Errors are expressed as ± SE.
[b] IMPDH I IC_{50}/IMPDH II IC_{50}.

Table 4
IC_{50} values for K562 cells proliferation and distribution coefficient of MPA and its derivatives

Compound	IC_{50}[a] (μM)	IC_{50} ratio[b]	Log D[c]
1	0.19 ± 0.01	16	−0.74
2	8.2 ± 0.5	48	−1.97
3	0.59 ± 0.04	0.14	−0.42
4	>50	n. d.	0.35
5	0.73 ± 0.04	0.52	3.31
6	3.9 ± 0.4	4.9	2.84
7	2.1 ± 0.2	5.0	2.21

n.d.: not determined.
[a] Errors are expressed as ± SE.
[b] K562 IC_{50}/IMPDH II IC_{50}.
[c] For calculation of distribution coefficient, the pH value (7.5) of the aqueous phase was input.

decrease in antimitotic activity.[21] On the other hand, replacement of 5-OMe by 5-OEt exhibited the same level of antiproliferative activity.[22] These results strongly suggested that **2** has very poor permeability. As shown in Figure 2, we propose resonance form of **2** in the culture medium. The pK_a values of 5- and 7-OH groups in **2** were estimated to be 7.96 and 8.91, respectively. We speculate that demethylation of the 5-OMe group can extend the pi-conjugated system of the chromophore to boost hydrophilicity of **2** (Log D = −1.97).

Although derivatives **3** and **5** were 2-orders of magnitude less potent as inhibitors of IMPDH II than MPA, the compounds were only 3-fold less active (IC_{50} = 0.59 and 0.73 μM, respectively) against proliferation of K562 cells than MPA (IC_{50} = 0.19 μM). These results suggested two hypothesis, one is that **3** and **5** perturb specific function(s) of other target molecule(s), another prevailing hypothesis is that the compounds are converted into MPA by esterase during incubation.

The IC_{50} ratios (K562 IC_{50}/IMPDH II IC_{50}) of **6** and **7** were 3-fold smaller than that of MPA. The partition coefficients of **6** (Log D = 2.84) and **7** (Log D = 2.21) are indicated that these compounds are hydrophobic in contrast with MPA (Log D = −0.74). Therefore, **6** and **7** could show higher permeability to pass through the plasma membrane of K562 cell than MPA. In the previous SAR studies on MPA, no discussion about relationship between bioactivity and hydrophobic property has been conducted. We propose that Log D of the compounds are important for SAR investigation of MPA analogues.

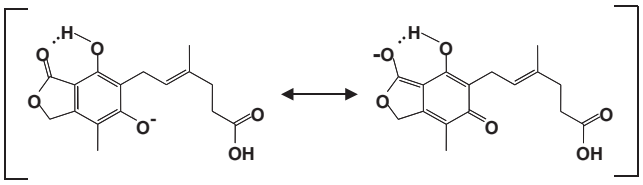

Figure 2. Model of resonance form of derivative **2**.

Table 5
K562 cell differentiation by MPA and its derivatives

Compound	Relative hemoglobin/genomic DNA[a]								
	0.125	0.25	0.5	1	2	4	8	16	32 (μM)
1	1.1	2.0	2.7	3.6	5.4	5.5	–	–	–
2	–	–	–	1.4	1.1	1.1	1.3	1.8	3.1
3	–	–	1.8	2.9	4.2	5.3	6.0	–	–
4	–	–	–	–	1.2	1.1	1.1	1.4	1.0
5	–	1.0	1.0	2.1	3.5	4.9	5.6	–	–
6	–	–	–	–	0.7	1.3	2.5	3.8	4.7
7	–	–	–	0.8	1.0	2.9	3.2	1.8	1.7

[a] Values of relative hemoglobin/genomic DNA are expressed as the ratios to non-treated K562 cells.

Next, we examined inhibitory effect of these compounds on intracellular IMPDH activity. When K562 cells, originally derived from a patient with chromic myelogenous erythroleukaemia, were cultured in the presence of IMPDH inhibitors or HDACs inhibitor, the cells undergo late erythroblast and acquire the capability to synthesize hemoglobin.[5,19,23] Pankiewicz and co-workers previously reported that compound **7** converted 1.6-fold larger percent (75%) of K562 cells into erythroblasts than MPA (46.4%) at the same concentration of 0.25 μM. The percent of differentiated K562 cells was determined by counting with microscopy after the benzidine staining in the past study.[5] We used a novel experimental method, in which differentiation of K562 cells was quantified by absorbance of the oxidation reaction products of *o*-dianisidine as described in Materials and Methods. In contrast to previous report, all MPA derivatives showed less potent inducing activity than MPA (Table 5). Over fivefold increasing of hemoglobin level (relative Hb/DNA) appeared in treatment with MPA at the concentration of 2 and 4 μM. The inductive effectiveness of

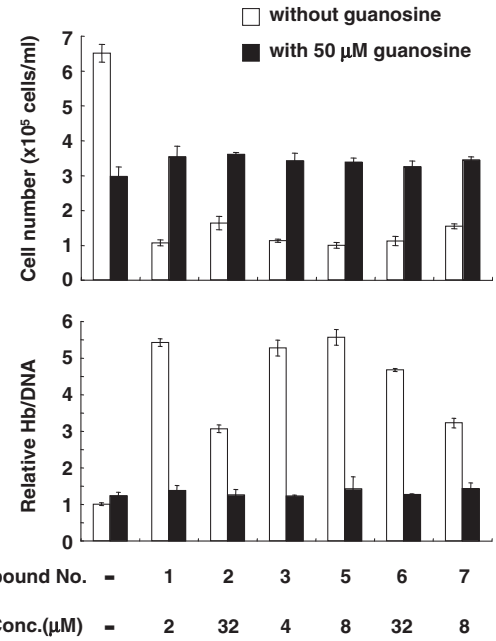

Figure 3. Effects of guanosine on antiproliferation and differentiation induction by MPA and its derivatives. Relative Hb/DNA indicates the expression level of hemoglobin as differentiation marker. Assays were performed as described in "Section 2". The data shown represent the mean ± SD of triplicate assay. Similar results were obtained in four separate experiments.

these compounds is expressed as follow: MPA > **3** = **5** > **7** > **6** > **2** > **4**. SAR of inductive activities was similar to that of antiproliferation activity.

To test whether MPA derivatives target IMPDH in cultured cells as in the case of MPA, we evaluated the effects of guanosine on cell growth suppression and cell differentiation by MPA derivatives (Fig. 3). IMPDH inhibitor blocks de novo GMP biosynthesis pathway, while guanosine can attenuate depletion of GMP pool via salvage pathway. The addition of guanosine alone unexpectedly suppressed cell proliferation to nearly half numbers (Fig. 3, upper graph). However, neither cell differentiation induction nor cytotoxic activity was observed by the addition of guanosine at the

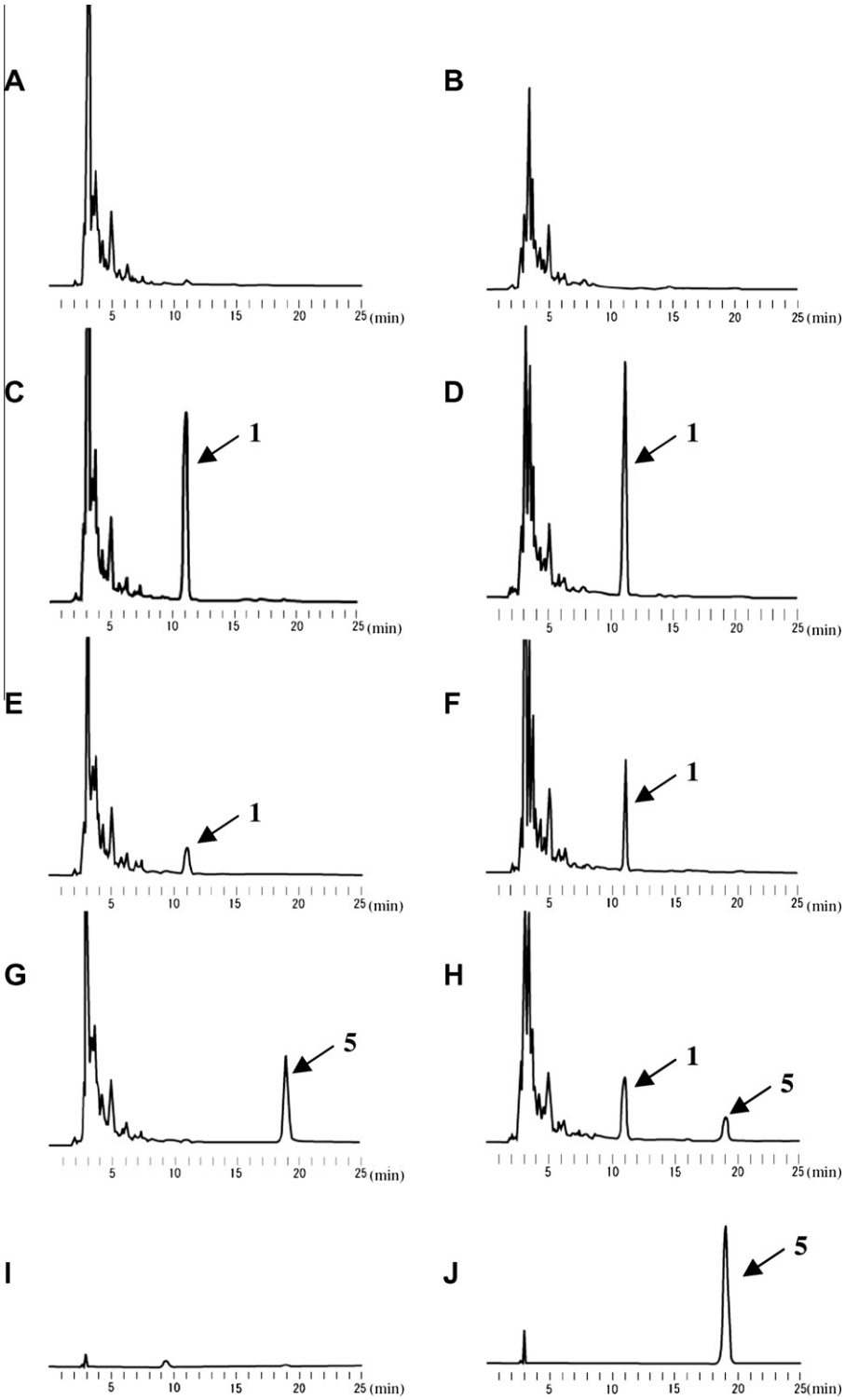

Figure 4. HPLC profiles of $CHCl_3$ extracts from K562 cell culture incubated with compound **3** and **5**. DMSO (panel **A** and **B**) or DMSO plus 20 nmol of MPA (**C** and **D**), **3** (**E** and **F**) or **5** (**G** and **H**) was incubated with K562 cells in culture medium at 37 °C for 0 (**A**, **C**, **E** and **G**) or 72 (**B**, **D**, **F**, and **H**) hour. MPA and its derivatives were extracted with $CHCl_3$ at pH 4.0. One-fifth of extracts (20 μl) in methanol were loaded onto the HPLC system. Twenty microliter of 200 μM **3** (**I**) and **5** (**J**) in methanol were loaded as reference. Analyses were performed as described in 'Section 2'. In panels, *X*-axis shows retention time and *Y*-axis shows absorbance at 305 nm. Arrows indicate peaks of MPA (**1**) and **5**.

Table 6
Conversion of compounds **3** and **5** to MPA by incubation with K562 cells in medium

Additive substrate	Incubation time	Relative MPA amount[a]
MPA	0 day	100
3	0 day	12.0
5	0 day	n.d.[b]
MPA	3 days	108
3	3 days	39.2
5	3 days	34.7

Analyses were performed as described in 'Section 2'.
[a] The relative amount was calculated from the area ratio of each peak to the peak area of MPA on HPLC profile (Fig. 4).
[b] n.d.: not detected.

concentration of 50 μM. As shown in Figure 3, attenuation of the effects of these compounds appeared in the present of 50 μM guanosine. These results suggest that **1**–**3**, and **5**–**7** show their bioactivities as a result of inhibition of IMPDH. The IC_{50} values of antiproliferative activities of **3** and **5** were smaller than those of the inhibitory activities against IMPDH (Table 3 and 4). Therefore, we assumed that the ester bond at C-7 and C-6′ position of **3** and **5** were hydrolyzed by esterase in the cells during incubation, and converted into MPA as an active form against K562 cells. To prove this hypothesis, the enzymatic conversion of **3** and **5** to MPA was conducted in K562 cell culture (Fig. 4 and Table 6). MPA and its derivatives were incubated with K562 cells in culture medium for 0–72 h and then extracted with $CHCl_3$ at pH 4.0. Figure 4 exhibits HPLC profile of the $CHCl_3$ extracts. The retention times of MPA and **5** were 11 and 19 min, respectively (Fig. 4A, C and J). Compound **3** could not be detected under the same HPLC conditions, probably because **3** has different UV absorption and optimum wave length for detection is not 305 nm (Fig. 4I). After incubating **3** in the culture medium for 3 days, a peak of MPA obviously appeared (Fig. 4E and F), whereas amount of **5** on 3 day decreased to quarter and the peak of MPA increased (Fig. 4G and H). These results clearly indicated that enzymatic hydrolysis of **3** and **5** occurred in the cell culture medium. As shown in Table 6, 39.2% and 34.7% of **3** and **5** were converted into MPA after incubation for 3 days, respectively. These observations are consistent with Nelson's remark that MPA derivatives such as 7-*O*-acetyl-6′-solketal ester, 6′-morpholinoethyl ester and 7-*O*-acetyl-6′-morpholinoethyl ester were converted to MPA in vivo.[24]

It is already shown that **7** (MPHA), 7-*O*-acetyl MPHA, 7-*O*-lauroyl MPHA, and 7-*O*-methyl MPHA act as tubulin-specific HDAC[s] inhibitors in cultured cell. Among them, **7**, 7-*O*-acetyl MPHA, and 7-*O*-lauroyl MPHA were potent inhibitor against cell proliferation. However, 7-*O*-methyl MPHA was inactive against cell growth (IC_{50}: 50 μM), whereas the compound is active against HDACs (IC_{50}: 0.9 μM).[11] Tables 3 and 4 exhibit that **4** does not have potent inhibitory effect on IMPDH and cell proliferation. Moreover, MPA did not show inhibitory activity against HDACs as previously described.[5,11] These results suggest that 7-*O*-acetyl MPHA, and 7-*O*-lauroyl MPHA are degraded into MPHA under the culture conditions, and then acquired inhibitory potency against IMPDHs and HDACs that results in inhibition of cell proliferation. Although maximal increasing of hemoglobin level by compound **7** (at 8 μM) reached over threefold of control (without treatment), **7** was less active than MPA, which induced 5-fold higher level of hemoglobin (Table 5 and Fig. 3).

In this paper, we exhibited that (i) functional groups at C-5, C-7, and C-6′ positions in MPA were important for inhibitory activity against IMPDH, (ii) it was difficult to improve specificity for IMPDH II by modification of 5-, 7-, and 6′-group, (iii) demethylation of 5-OMe resulted in increasing hydrophilicity, and lowering cell permeability, (iv) ester bond of protective group at C-7 or C-6′ position was hydrolyzed in the cell culture medium, (v) the effects of tubulin-specific HDAC[s] inhibitor (**7**) on proliferation and differentiation were less potent than its inhibitory activity against IMPDH. The present work may provide insight into the development of new class of drug leads for treating cancer and transplantation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.004.

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