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Derivatives of *Dictyostelium* differentiation-inducing factors inhibit lysophosphatidic acid–stimulated migration of murine osteosarcoma LM8 cells

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ABSTRACT

Osteosarcoma is a common metastatic bone cancer that predominantly develops in children and adolescents. Metastatic osteosarcoma remains associated with a poor prognosis; therefore, more effective anti-metastatic drugs are needed. Differentiation-inducing factor-1 (DIF-1), −2, and −3 are novel lead anti-tumor agents that were originally isolated from the cellular slime mold *Dictyostelium discoideum*. Here we investigated the effects of a panel of DIF derivatives on lysophosphatidic acid (LPA)-induced migration of mouse osteosarcoma LM8 cells by using a Boyden chamber assay. Some DIF derivatives such as Br-DIF-1, DIF-3(+2), and Bu-DIF-3 (5–20 μM) dose-dependently suppressed LPA-induced cell migration with associated IC_{50} values of 5.5, 4.6, and 4.2 μM, respectively. On the other hand, the IC_{50} values of Br-DIF-1, DIF-3(+2), and Bu-DIF-3 versus cell proliferation were 18.5, 7.2, and 2.0 μM, respectively, in LM8 cells, and >20, 14.8, and 4.3 μM, respectively, in mouse 3T3-L1 fibroblasts (non-transformed). Together, our results demonstrate that Br-DIF-1 in particular may be a valuable tool for the analysis of cancer cell migration, and that DIF derivatives such as DIF-3(+2) and Bu-DIF-3 are promising lead anti-tumor agents for the development of therapies that suppress osteosarcoma cell proliferation, migration, and metastasis.

1. Introduction

Osteosarcoma is a common primary malignant bone tumor that predominantly develops in children and adolescents. The current strategy for the treatment of high-grade osteosarcoma involves surgical tumor resection and adjuvant chemotherapy. However, the presence of metastases at diagnosis continues to be associated with a poor prognosis and the currently approved drugs exhibit major adverse effects [1,2]. Therefore, improved strategies for the treatment of osteosarcoma and metastatic osteosarcoma are required.

Differentiation-inducing factor-1 (DIF-1), −2, and −3 (Fig. 1A) are inducers of stalk cell differentiation in the cellular slime mold *Dictyostelium discoideum* [3–6]. It has recently been shown that DIF-1 and DIF-2 also function as chemotactic modulators during *D. discoideum* development [7]. DIFs have also been shown to possess anti-tumor activities; that is, they have been shown to suppress cell proliferation and induce or promote cell differentiation in the mouse erythroleukemia B8, rat pancreatic AR42J, human myeloid leukemia K562, and human promyelocytic leukemia HL-60 cell lines; certain human gastric cancer cell lines; and human epithelial carcinoma HeLa cells [8–15]. We previously found that DIF-3 and several chemically synthesized DIF-3 derivatives possess potent anti-proliferative activity in K562 and HeLa cells (Fig. 1B) [11,13,14,16]. Despite DIF derivatives being promising lead anti-tumor agents, their effects on cancer invasion and metastasis have not yet been sufficiently investigated.

Abbreviations: DIF, differentiation-inducing factor; LPA, lysophosphatidic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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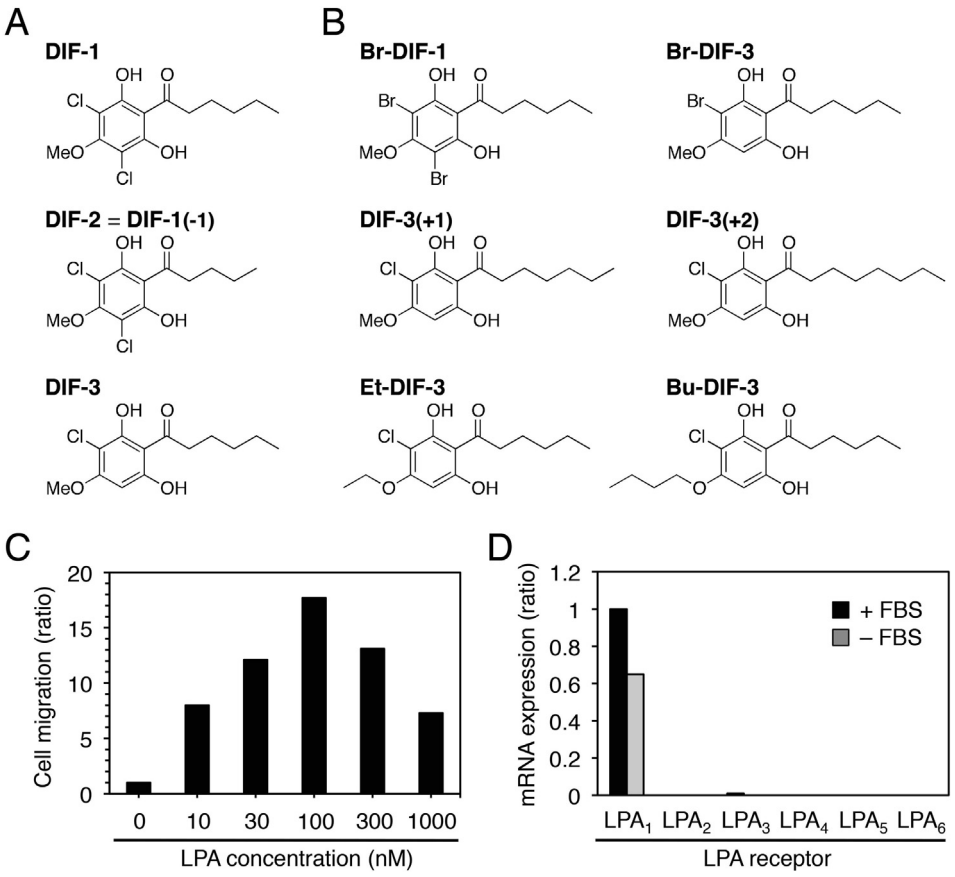

Fig. 1. Chemical structures of DIF derivatives (A, B) and analysis of lysophosphatidic acid (LPA)-induced migration of LM8 cells (C, D). (A) Chemical structures of naturally occurring DIFs. DIF-1 (1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one), DIF-2 (1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)pentan-1-one), and DIF-3 (1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one) are endogenous factors in *D. discoideum*. DIF-2 is sometimes referred to as DIF-1(-1) in this study for the sake of convenience. The order of differentiation-inducing activity in *D. discoideum* is DIF-1 > DIF-2 >> DIF-3 [26,27], whereas the order of anti-proliferative activity in tumor cells is DIF-3 > DIF-1 > DIF-2 [11,16]. (B) Chemical structures of six of the artificial DIF derivatives tested in this study. The chosen derivatives include derivatives that had undergone halogen substitution, had longer alkyl chains at the acyl group, or had larger alkyl groups in place of the methyl group. (C) Cell migration was assessed with a Boyden chamber in the presence or absence of the indicated concentrations of LPA in the lower chambers. Serum-starved cells were incubated for 4 h in the upper chambers, and the number of cells migrated to the lower chambers was counted. Relative values of migrated cells are shown. (D) LPA receptors expressed in LM8 cells. RNAs were collected from cells incubated in MEM-α-FBS (+FBS) or serum-free medium (−FBS), and real time reverse transcription–quantitative polymerase chain reaction was performed to assess the expressions of LPA receptor and GAPDH mRNA. Expression levels of the LPA receptor mRNAs were normalized to the expression of GAPDH mRNA and the relative expression levels are shown.

In the present study, by using a cancer invasion (cell migration) assay system and murine LM8 cells, which is a highly metastatic osteosarcoma cell line [17], we examined the effects of a panel of DIF derivatives on lysophosphatidic acid (LPA)-induced migration of LM8 cells *in vitro*. Our results show that certain DIF derivatives significantly suppressed LPA-induced LM8 migration, which may be lead anti-tumor agents for the development of therapies that suppress osteosarcoma cell proliferation, migration, and metastasis.

2. Materials and methods

2.1. Cells and reagents

Murine osteosarcoma LM8 cells [17] were grown and maintained at 37 °C (5% CO_2 in air) in Modified Eagle's Medium (MEM)-α (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS). Murine 3T3-L1 fibroblasts and human cervical cancer HeLa cells were grown and maintained at 37 °C (5% CO_2 in air) in Dulbecco's Modified Eagle's Medium (DMEM) (D5796; Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% FBS. Both media also contained 75 μg/ml penicillin and 50 μg/ml streptomycin.

Lysophosphatidic acid (LPA) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Fatty acid-free bovine serum albumin (BSA) was purchased from Calbiochem-Novabiochem (San Diego, CA, USA). DIF derivatives were synthesized as described previously [16], dissolved in ethanol (EtOH) or dimethyl sulfoxide (DMSO), and stored at −20 °C.

2.2. Cell migration assay

LM8 cells were grown in 90-mm plastic dishes in MEM-α-FBS and then incubated overnight in serum-free medium (MEM-α supplemented with 0.1% [w/v] BSA). The cells were then trypsinized (harvested), washed twice and resuspended in the serum-free medium (10^6 cells/ml), and used in the cell migration assay.

Cell migration was assessed by using a blind Boyden chamber (Neuro Probe Inc., Gaithersburg, MD, USA) as previously reported [18–20]. Briefly, the lower chambers were filled with approximately 160 μl of serum-free medium containing vehicles, 0.1% EtOH or DMSO, or various concentrations of one of DIF derivatives (usually, 10 μM) and/or LPA (usually, 100 nM). The chambers were then covered with an 8-μm-pore membrane filter (Neuro Probe Inc.) pre-coated with type I collagen. The upper chambers were filled with 45 μl of serum-free medium containing

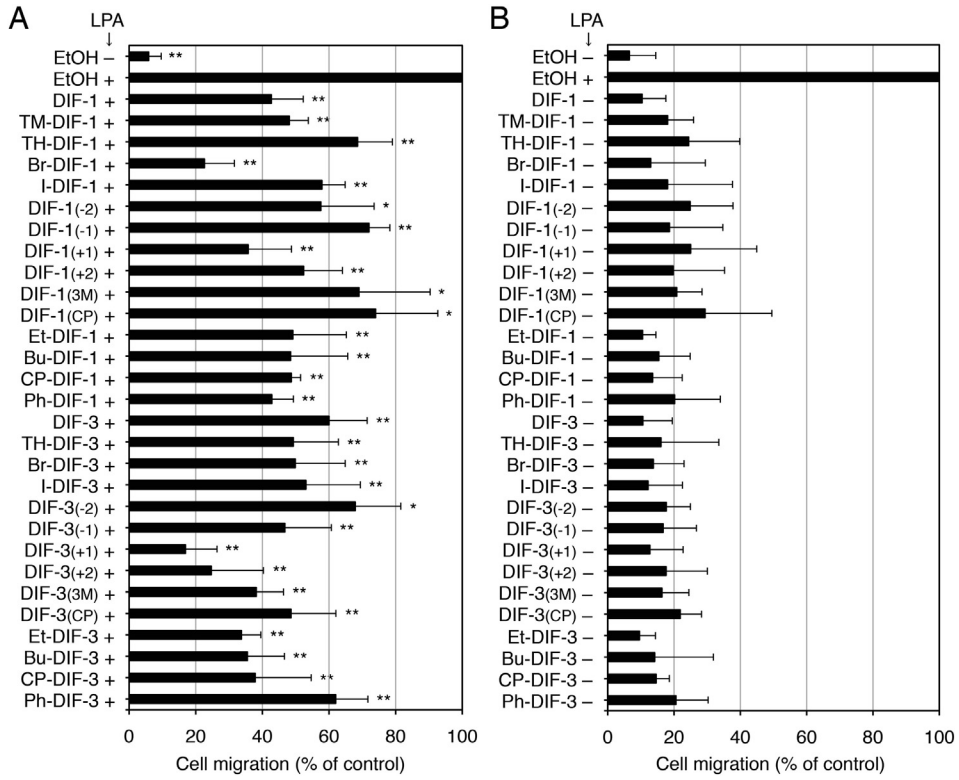

Fig. 2. Effects of DIF derivatives on LPA-induced cell migration in LM8 cells. Serum-starved cells were stimulated with LPA (100 nM) in the presence of 0.1% EtOH (vehicle) or 10 μM of one of the DIF derivatives in a Boyden chamber. Cells were incubated for 4 h and the number of cells migrated from the upper chambers to the lower chambers was counted. The indicated DIF derivatives were present in the upper and the lower chambers (A) or only in the lower chambers (B). Mean values and SD (bars) of relative numbers of migrated cells from several independent experiments (A: n = 3–8; B: n = 2–5) are shown. $^{*}P < 0.05$; $^{**}P < 0.01$ versus EtOH plus LPA control.

2 × concentration of vehicle or one of DIF derivatives, and then 45 μl of LM8 cell suspension (4.5×10^4 cells/chamber). After incubation for 4 h at 37 °C (5% CO_2 and 95% air), the number of cells that had migrated to the lower surface of the membrane filter were counted by means of microscopic observation; four microscopic fields were examined per sample.

To determine the 50% inhibitory concentration (IC_{50}) of each compound versus cell migration, various concentrations (1–20 μM) of the DIF derivatives were used. Relative inhibition was determined for each concentration for each compound, and IC_{50} values were determined from the dose–response curves.

2.3. Cell proliferation assay

LM8, 3T3-L1, or HeLa cells were incubated for 3 days in 12-well plates at 2.5×10^3 cells/well (LM8) or 5×10^3 cells/well (3T3-L1 and HeLa), with each well containing 1 ml of MEM-α-FBS (LM8) or DMEM-FBS (3T3-L1 and HeLa) in the presence of 10 μM of one of the DIF derivatives or 0.2% (v/v) EtOH. The incubation medium was then discarded and the cells incubated with 1 ml of fresh medium containing 5% (v/v) Alamar blue (a cell number indicator; Wako Pure Chemical Industries, Osaka, Japan) until the color changed. Relative cell number was assessed by measuring absorbance at 570 nm (reference at 595 nm) as described previously [11,15,21].

To determine the IC_{50} of each compound versus cell proliferation, cells were cultured for 3 days in the presence of various concentrations of each compound. Relative cell numbers were determined by using Alamar blue, and the IC_{50} value was determined from the dose–response curve drawn with the average values of three independent experiments.

Table 1
Comparison of the effects of DIF derivatives on cell migration and cell proliferation.

Compound	IC_{50} (μM) vs. CM	IC_{50} (μM) vs. CP		
	LM8	LM8	3T3-L1	HeLa
DIF-1	8.5	18.2	>20	>20
DIF-2	>20	>20	>20	>20
DIF-3	10.2	15.5	>20	16.2
Br-DIF-1	5.5	18.5	>20	14.0
Br-DIF-3	10.0	14.5	>20	16.8
DIF-3(+1)	5.1	7.8	16.0	11.5
DIF-3(+2)	4.6	7.2	14.8	8.3
Et-DIF-3	6.6	7.4	11.2	9.5
Bu-DIF-3	4.2	2.0	4.3	3.2

IC_{50} values versus LPA-induced cell migration (CM) of LM8 cells and cell proliferation (CP) of LM8, 3T3-L1, and HeLa cells.

2.4. Real time RT-qPCR (reverse transcription–quantitative polymerase chain reaction) assay for LPA receptor expression

LM8 cells were incubated in two 90-mm dishes; the cells in one dish were incubated for three days with 10 ml of MEM-α-FBS, while the cells in the other dish were incubated for two days with 10 ml of MEM-α-FBS and then incubated overnight with 10 ml of serum-free medium. The cells in the two dishes were washed with PBS and collected with TRI Reagent (Sigma–Aldrich), and total RNA was isolated from the cells according to the manufacturer's instructions. After treatment with DNase I (Promega, Madison, WI, USA) to remove any trace genomic DNA present in the RNA preparations, 5 μg of total RNA was reverse transcribed by using random priming and Multiscribe reverse transcriptase according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA).

To evaluate the expression of LPA receptor (LPA_{1-6}) mRNA, RT-qPCR was performed by using TaqMan Real-Time technology and a Sequence Detection System (7500 Fast; Applied Biosystems); the probes specific for mouse LPA_1 (product ID: Mm00439145), LPA_2 (Mm00469562), LPA_3 (Mm00469694), LPA_4 (Mm01228532), LPA_5 (Mm01190818), LPA_6 (Mm00613058), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 4352932E) were obtained from TaqMan gene expression assay kits (Life Technologies). Other experimental conditions were as described previously [22]. The expression levels of the LPA receptor mRNAs were normalized to the expression of GAPDH mRNA, and relative expression levels were calculated.

2.5. Measurement of mitochondrial oxygen consumption

Mitochondria-enriched fraction was prepared from mouse liver, and mitochondrial oxygen consumption was monitored by using a Clark-type oxygen electrode (Strathkelvin Instruments Ltd., North Lanarkshire, Scotland) as described previously [23,24]. The mitochondria-enriched fraction was incubated at 30 °C in oxygen measurement buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 0.1 mM EDTA, 3 mM phosphate, 5 mM succinate, 5 mM glutamate, and 20 mM Tris–HCl pH 7.4) in the presence of 1% (v/v) DMSO (vehicle) or various concentrations of one of the DIF derivatives or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a mitochondrial decoupler. After recording State 4 of the respiration reaction, an aliquot of adenosine diphosphate (ADP) was added to a final concentration of 200 μM to induce State 3 respiration.

2.6. Statistics

Student's *t* test (two-tailed, unpaired) was performed for the statistical analyses. *P* values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. LPA-induced migration of LM8 cells

LPA is a simple, natural, bioactive phospholipid that induces cell migration, invasion, and metastasis in a variety of tumor cells [25]. We began by investigating whether or not LPA induces cell migration in LM8 cells and which species of LPA receptor are expressed by LM8 cells. We found that LPA at 10–1000 nM (optimal concentration: 100 nM) induced cell migration (Fig. 1C) and that LPA_1 was the dominantly expressed receptor in LM8 cells under both serum-present and serum-starved conditions (Fig. 1D).

3.2. Effects of DIF derivatives on LPA-induced cell migration and proliferation

We next examined the effects of DIF derivatives on LPA-induced cell migration in LM8 cells. All of the DIF derivatives at 10 μM significantly suppressed LPA-induced cell migration (Fig. 2A), whereas none of the DIF derivatives significantly affected cell migration in the absence of LPA (Fig. 2B). Among the DIF derivatives examined, Br-DIF-1, Br-DIF-3, DIF-3(+1), DIF-3(+2), Et-DIF-3, and Bu-DIF-3 were the most active in inhibiting LPA-induced cell migration. IC_{50} values of the DIF derivatives against LPA-induced cell migration in LM8 cells are shown in Table 1.

We next examined the effects of DIF derivatives on cell growth (proliferation) in LM8 cells (Fig. 3) and mouse 3T3-L1 fibroblasts, which is a model of non-transformed cells (Fig. 3). In both LM8 cells and 3T3-L1 fibroblasts, some of the DIF derivatives examined strongly suppressed cell proliferation (i.e., DIF-3(+1), DIF-3(+2), Et-DIF-3, Bu-DIF-3, CP-DIF-3, and Ph-DIF-3); however, overall, the anti-proliferative effects of the DIF derivatives were weaker in 3T3-L1 fibroblasts compared with in LM8 cells. IC_{50} values of several

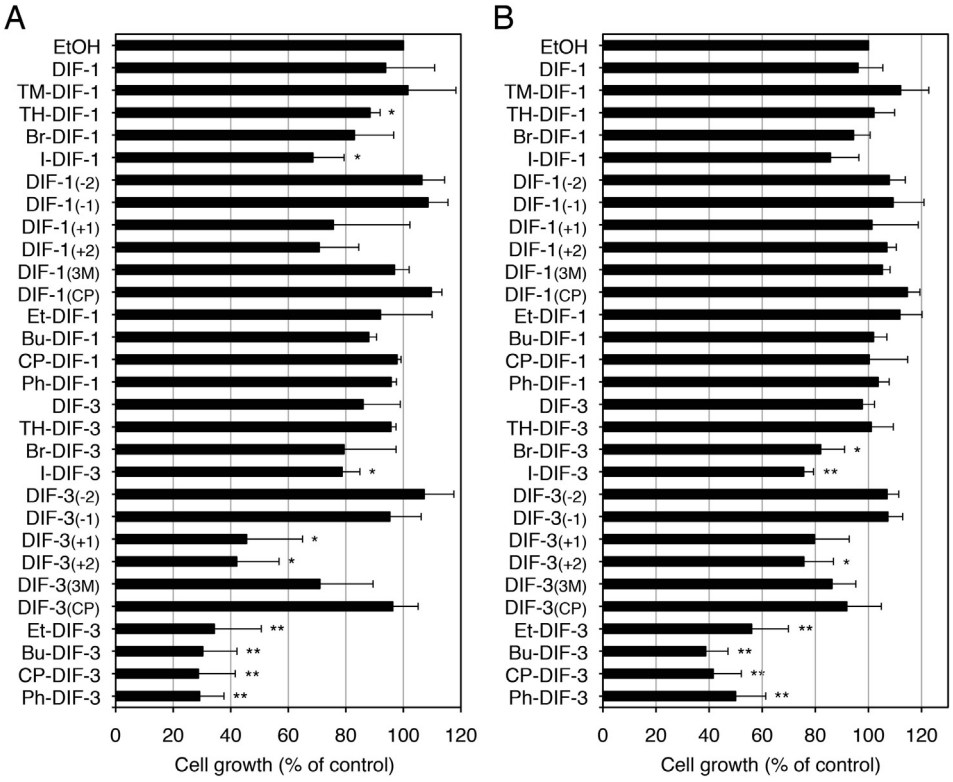

Fig. 3. Effects of DIF derivatives on cell proliferation (growth) in LM8 cells and 3T3-L1 fibroblasts. LM8 cells (A) and 3T3-L1 fibroblasts (B) were incubated for 3 days with 0.1% EtOH or 10 μM DIF derivatives and relative cell number was assessed. Mean values and SD (bars) of three independent experiments are shown. $^{*}P < 0.05$; $^{**}P < 0.01$ versus EtOH plus LPA control.

representative DIF derivatives, which included halogen-substituted derivatives, derivatives with longer alkyl chains at the acyl group, and derivatives with larger alkyl groups in place of the methyl group, versus LPA-induced cell migration in LM8 cells and cell growth in LM8, 3T3-L1, and human cervical cancer HeLa cells were also assessed (Table 1). It is noteworthy that Br-DIF-1, a potent inhibitor of cell migration in LM8, did not significantly suppress cell proliferation in either LM8 or 3T3-L1 cells, whereas DIF-3(+1), DIF-3(+2), Et-DIF-3, and Bu-DIF-3 significantly suppressed both cell migration and cell proliferation in LM8 cells (Fig. 2A, Table 1).

3.3. Effects of DIF derivatives on mitochondrial oxygen consumption

We previously found that DIF-3 and Bu-DIF-3, like CCCP, are potent mitochondrial uncouplers, that is, they promote mitochondrial oxygen consumption [21]. We therefore examined the effects of the DIF derivatives presented in Fig. 1 on oxygen consumption in murine mitochondria-enriched fraction (Fig. 4A, B). The control compound, CCCP, at 0.1–2 μM, dose-dependently promoted mitochondrial oxygen consumption during State 4 respiration, and DIF-1 at 1–20 μM also promoted mitochondrial oxygen consumption in a dose-dependent manner (Fig. 4A). Mitochondrial oxygen consumption was promoted by all of the DIF derivatives tested (Fig. 4B), suggesting that the DIF derivatives inhibit LPA-induced cell migration, at least in part, by disturbing mitochondrial activity.

To further assess this possibility, we examined the effects of CCCP and azide (an inhibitor of electron transfer from cytochrome c to Complex IV) on LPA-induced LM8 cell migration (Fig. 4C). CCCP at 2–10 μM significantly inhibited cell migration in a dose-independent manner, although its effect was limited, and azide at 0.002%–0.010% did not strongly inhibit cell migration (Fig. 4C) despite the fact that 10 μM CCCP [21] and 0.01% azide (data not shown) each suppressed cell proliferation. These results suggest that DIF derivatives inhibit LPA-induced cell migration partly by disturbing mitochondrial activity, but that normal mitochondrial activity may not be necessary for LPA-induced cell migration.

4. Discussion

As described, osteosarcoma is the most common metastatic bone tumor predominantly occurring in children and adolescents. The patients with metastases have a poor prognosis and need novel anti-metastatic drugs.

DIFs 1–3 were originally identified as differentiation-inducing factors in *D. discoideum* [3–6]. DIF-1 and DIF-2 have since been shown to also modulate the chemotactic movement of *D. discoideum* cells toward cAMP [7]. Moreover, a number of DIFs and their derivatives have been shown to possess anti-proliferative and differentiation-inducing activities in several mammalian tumor cell lines [8–16]. However, it has not been verified if DIF derivatives possess anti-metastatic activity in cancer cells.

The results of the present study show that DIF derivatives significantly suppress LPA-stimulated cell migration (invasion) in murine osteosarcoma LM8 cells; since LPA possesses a diverse range of biological activities that are implicated in tumor cell migration, invasion and metastasis [25], LPA is an important compound worth investigating. Importantly, the DIF derivatives that inhibited LPA-induced LM8 cell migration also promoted mitochondrial oxygen consumption (Fig. 4A, B) [21], which suggests that these DIF derivatives inhibit LPA-induced LM8 cell migration by disturbing mitochondrial activity. However, since the

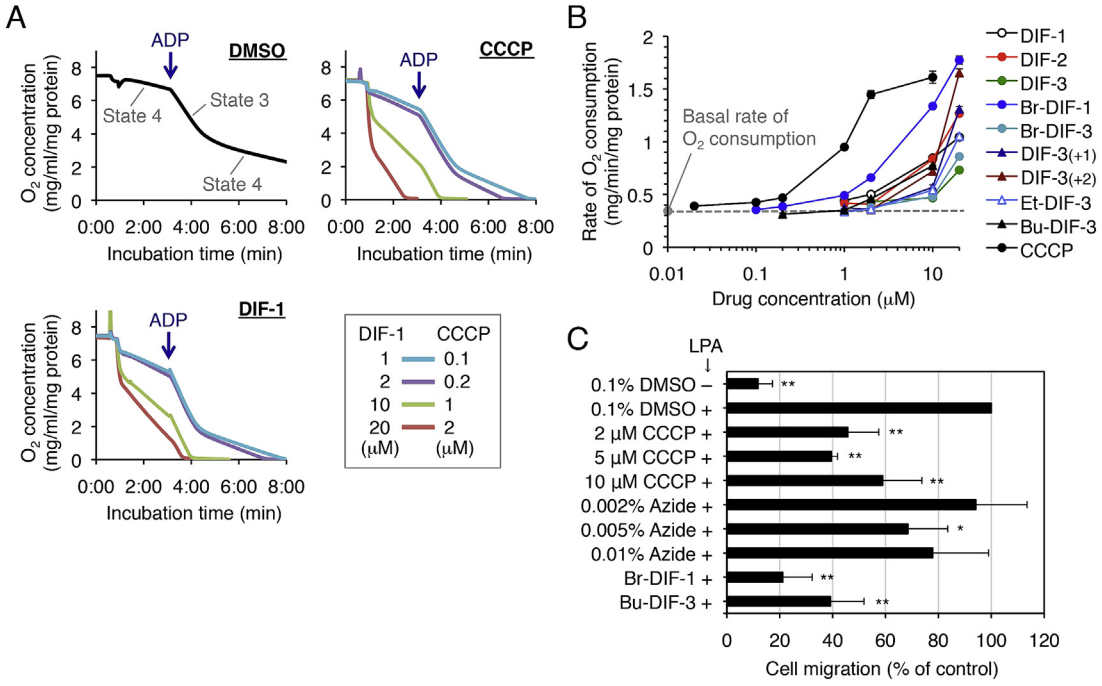

Fig. 4. Effects of DIF derivatives and the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on mitochondrial oxygen consumption and cell migration. (A, B) Mitochondria were prepared from mouse liver, and mitochondrial oxygen concentration was monitored in the presence of 1% DMSO (vehicle) or various concentrations of the indicated compounds. After recording the State 4 respiration reaction, adenosine diphosphate (ADP) (200 μM) was added to induce the State 3 respiration reaction. Data with DMSO, DIF-1, and CCCP are shown in (A). Rates of oxygen consumption during State 4 respiration were calculated and are presented as a function of drug concentration in (B). The basal rate of oxygen consumption (0.344 mg/min/mg protein) is indicated in the graph by a dotted gray line. (C) Effects of mitochondrial poisons on LPA-induced cell migration in LM8. Serum-starved cells were stimulated with LPA (100 nM) in the presence of 0.1% DMSO (vehicle) or the indicated concentrations of CCCP or azide in a Boyden chamber. Cells were incubated for 4 h and the number of cells that had migrated from the upper chambers to the lower chambers was counted. Mean values and SD (bars) of relative migrated cell numbers from three independent experiments (n = 3) are shown. $^{*}P < 0.05$; $^{**}P < 0.01$ versus DMSO control.

mitochondrial uncoupler CCCP at 2–10 μM did not dose-dependently inhibit LPA-induced LM8 cell migration, and azide up to 0.01% also did not inhibit cell migration, the involvement of the mitochondria in the inhibitory action of DIF derivatives on cell migration is likely limited.

Although the results of the present study do not reveal the precise mechanisms of the inhibitory effects of DIF derivatives on cell proliferation and migration in mammalian cells, it is likely that the mechanisms differ at least in part because 1) Br-DIF-1 inhibited LM8 cell migration (Fig. 2A) but did not inhibit cell proliferation of either LM8 and 3T3-L1 cells (Fig. 3); 2) DIF-3(+2) inhibited LM8 cell migration and proliferation (Figs. 2A and 3A) but did not inhibit 3T3-L1 cell proliferation (Fig. 3B); and 3) Bu-DIF-3 inhibited the migration of LM8 cells and proliferation of both LM8 and 3T3-L1 cells (Figs. 2A and 3). In addition, DIF-3(+2) and Bu-DIF-3 suppressed HeLa cell growth with IC_{50} values of 8.3 and 3.2 μM, respectively (Table 1).

In conclusion, DIF derivatives such as Br-DIF-1 may be valuable tools for the analysis of cancer cell migration, and DIF derivatives such as Bu-DIF-3 may be valuable tools for the analysis of mammalian cell growth and migration. DIF-3(+2) may be a good lead compound for the development of therapies that inhibit cancer cell growth, invasion, and metastasis with few adverse effects. Although the mechanism underlying the action of each DIF derivative remains to be precisely elucidated, the present results provide important insights into the pharmacological potential of DIF derivatives.

Conflict of interest

Patent related to this article issued October 10, 2014 in Japan (No. 5626755). Gunma University holds the Patent. YK, MK, HK and YO are inventors of the patent.

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