ELSEVIER

European Journal of Pharmacology 381 (1999) 57–62

ejp

www.elsevier.nl/locate/ejphar

Effects of differentiation-inducing factors of *Dictyostelium discoideum* on human leukemia K562 cells: DIF-3 is the most potent anti-leukemic agent

Yuzuru Kubohara *

Department of Molecular Physiology, Institute for Molecular and Cellular Regulation (IMCR), Gunma University, Showa-machi 3-39-15, Maebashi 371-8512, Japan

Received 18 March 1999; received in revised form 23 July 1999; accepted 30 July 1999

Abstract

DIF-1 (differentiation-inducing factor-1; 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one) is a putative morphogen that induces stalk cell formation in the cellular slime mold, *Dictyostelium discoideum*. It has been previously reported that DIF-1 exhibits anti-tumor activity in mammalian cells. In this study, we examined the effects of six DIF analogues on DNA synthesis, cell growth, erythroid differentiation, and cytosolic free calcium concentration ($[Ca^{2+}]_i$) in human leukemia K562 cells. The DIF analogues used here were DIF-1, DIF-2 (which has pentanone in place of hexanone), DIF-3 (dechlorinated form of DIF-1), 2-MIDIF-1 (2-methoxy isomer of DIF-1), DMPH (dechlorinated form of DIF-3), and THPH (4-hydroxy substitution of DMPH). DIF-3 proved to be the most potent anti-leukemic agent among them, and the order of potency for causing growth inhibition, erythroid induction, and increases in $[Ca^{2+}]_i$ was established as DIF-3 > DIF-1 > DIF-2 > DMPH > 2-MIDIF-1 > THPH in all the categories tested. The present results suggest new routes for the development of more potent and effective anti-tumor agents.

Keywords: *Dictyostelium*; Differentiation-inducing factor; Growth inhibition; Erythroid differentiation; Ca^{2+}

1. Introduction

The simple eukaryote, *Dictyostelium discoideum*, forms a fruiting body that consists of spores and a multicellular stalk at the end of its development. It has been shown that cAMP and chlorinated alkylphenones, called differentiation-inducing factors (DIFs), are required for stalk-cell differentiation (Bonner, 1970; Town et al., 1976; Kay et al., 1989). DIF-1, 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one (Fig. 1) (Morris et al., 1987), is the most active form of the DIFs (Fig. 2), and is suggested to function at least in part via an increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$) in *D. discoideum* (Kubohara and Okamoto, 1994; Schaap et al., 1996; Azhar et al., 1997).

DIF-2, 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)pentan-1-one (Fig. 1) (Morris et al., 1988), is the second most active form of naturally occurring DIFs (Fig. 2), and it has been suggested that this molecule has some role(s) during the early development of *D. discoideum* other than stalk-cell induction (Wurster and Kay, 1990). DIF-3, 1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl) hexan-1-one (Fig. 1), is the first metabolite produced by living *Dictyostelium* cells from DIF-1 (Morandini et al., 1995) and has only 4% of the stalk-cell-inducing activity of DIF-1 (Fig. 2). Two artificial DIF analogues, 2-MIDIF-1 (2-methoxy isomer of DIF-1) and THPH (1-(2,4,6-trihydroxyphenyl)hexan-1-one), are not active in stalk-cell induction (Figs. 1, 2). DMPH (1-(2,6-dihydroxy-4-methoxyphenyl)hexan-1-one) is probably also a non-active form in *D. discoideum* (Figs. 1, 2).

Recently, DIF-1 was found to suppress cell growth and to induce erythroid differentiation in murine (B8) and human (K562) leukemia cells (Asahi et al., 1995). It has also been shown that DIF-1 increases $[Ca^{2+}]_i$ and suppresses cell growth in rat pancreatic tumor AR42J cells and human myeloid leukemia HL-60 cells (Kubohara, 1997; Kubohara et al., 1995a,b), and that DIF-1 promotes retinoic acid-induced cell differentiation in HL-60 cells

* Tel.: +81-27-220-8847; fax: +81-27-220-8849; e-mail: kubohara@akagi.sb.gunma-u.ac.jp

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00548-8

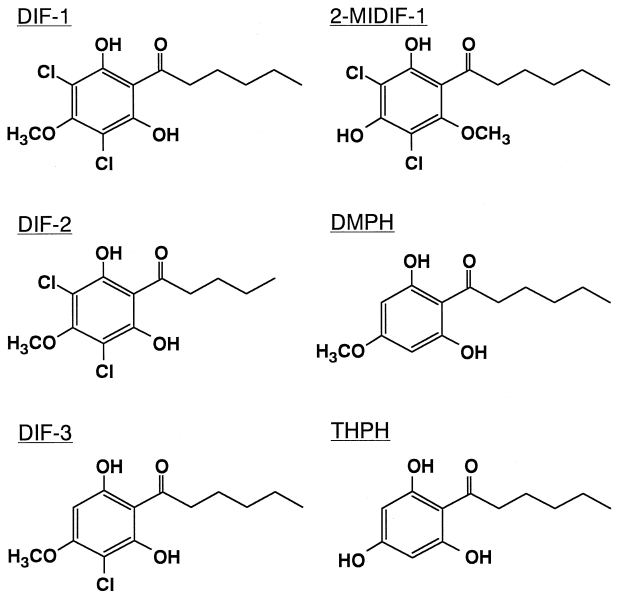

Fig. 1. Chemical structure of DIF analogues. DIF-1; 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one. DIF-2; 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)pentan-1-one. DIF-3; 1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one. 2-MIDIF-1; 2-methoxy isomer of DIF-1. DMPH; 1-(2,6-dihydroxy-4-methoxyphenyl)hexan-1-one. THPH; 1-(2,4,6-trihydroxyphenyl)hexan-1-one.

(Kubohara, 1997). It is thus expected that DIF-1 or its artificial analogues can be used in chemoprevention/differentiation therapy against some sorts of cancer. However, the study of DIF-1 in the field of tumor biology has started only recently, and many hurdles remain to be cleared before DIF-1-like molecules can be used therapeutically.

In this study, in order to evaluate the structure–effect relation of DIF-1-like molecules and develop more potent anti-tumor drugs, we examined the in vitro effects of six DIF analogues (Fig. 1) on DNA synthesis, cell growth, cell differentiation and $[Ca^{2+}]_i$ in human leukemia K562 cells. We show that DIF-3 is the most potent anti-leukemic agent and discuss the chemical structure–effect relation.

2. Materials and methods

2.1. Cell line and chemicals

Human leukemia K562 cells were used in this study. DIF-1 was purchased from Affiniti Research Products (UK). DIF-2, DIF-3, and DMPH were kindly synthesized by a chemical company after the method of Masento et al. (1988). 2-MIDIF-1 and THPH were kind gifts from Dr. R.R. Kay (MRC Laboratory of Molecular Biology, Cambridge, UK). All the DIF analogues were stored at −20°C as 10 mM solutions in ethanol. Alamar Blue (cell number indicator) and fluo-3/AM were obtained from Wako (Osaka, Japan).

2.2. Cell culture and assay for cell growth and erythroid differentiation

K562 cells were maintained at 37°C (5–8% CO_2) in tissue culture dishes filled with growth medium (RPMI1640 medium with 10% heat-inactivated fetal bovine serum, 25 mg/l penicillin, and 50 mg/l streptomycin; designated RPMI).

To examine the effects of DIF analogues on cell growth and erythroid differentiation, K562 cells were incubated in multi(12)-well plates, with each well containing 1 ml of RPMI (5×10^4 cells/ml) supplemented with or without the reagents. On Day 3, 400 μl of the sample cell suspensions were transferred into multi(24)-well plates (the remaining 600 μl of the samples in the 12-well plate were further incubated for the assay for erythroid differentiation on Day 5), and 1/20 volume (20 μl) of Alamar Blue was added to each well. After a 1–2 h incubation at 37°C (5–8% CO_2), 100 μl of the sample solution were transferred to a 96-well plate and absorbance at 570 nm (reference at 595 nm) was measured with a Microplate Reader (Bio-Rad, Model 550). Cell number is given as a ratio of the absorbance (control = 100). On Day 5, the rest of the samples (600 μl each) was used for the assay for erythroid differentiation; 60 μl of diaminobenzidine (DAB) solution were added to each well, and the samples were left to stand at room temperature for 30–60 min. Benzidine-stained cells (hemoglobin-producing cells) were counted with a microscope. The DAB solution was prepared just before use, by mixing the stock DAB (3% (w/v) DAB in 90% acetic acid), H_2O and H_2O_2 (31% solution) at 1:5:1 (Asahi et al., 1995). It should be noted that, in order to observe the clearest difference in anti-leukemic potential of the DIF analogues, we examined the effects of the analogues on cell growth and cell differentiation on Days 3 and 5, respectively.

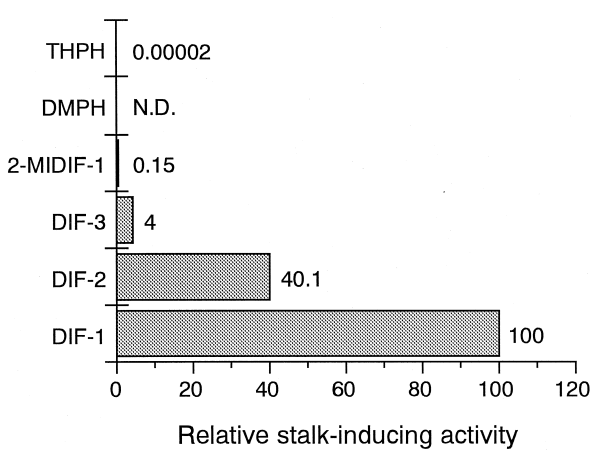

Fig. 2. Relative stalk-differentiation-inducing activity of DIF analogues. Data are from previous reports (Masento et al., 1988; Wurster and Kay, 1990). ND: not determined.

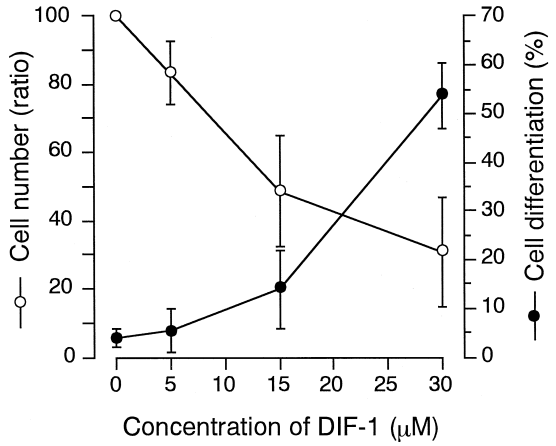

Fig. 3. Effects of DIF-1 on cell growth and erythroid differentiation in K562 cells. K562 cells were incubated at 5×10^4 cells/ml without or with 5–30 μM DIF-1. On Day 3, the relative cell number was optically measured with Alamar Blue as described under Section 2 (control cell number = 100). On Day 5, cells were stained with diaminobenzidine, and the stained cells were counted microscopically. Data are given with the means ± S.D. of three independent experiments ($n = 3$).

2.3. Measurement of DNA synthesis

DNA synthesis was observed by measuring 5-bromo-2′deoxy-uridine (BrdU) incorporation using a BrdU labeling and detection kit III (Boehringer Mannheim, Germany). Briefly, K562 cells were incubated for 2 h at 37°C (5% CO_2) in multi(96)-well plates (4×10^4 cells/well), with each well containing 100 μl of RPMI supplemented with or without 10–60 μM DIF analogues. (Prior to the cell incubation, the wells were coated with poly-D-lysine; 50 μl of poly-D-lysine (0.2 mg/ml) solution were added to each well and incubated for 1 h at room temperature. The poly-D-lysine solution was aspirated, and the wells were washed with RPMI and used for cell incubation.) Ten microliters of BrdU-labeling reagent were added to each well and incubated for 2 h at 37°C (5% CO_2). Cells were precipitated by centrifugation ($350\times g$, 10 min), and the supernatants were aspirated carefully. Cells were fixed with 70% ethanol (0.5 M HCl) and used for the assay for BrdU incorporation according to the manual. Finally, the quantity of BrdU taken up by the cells was detected by measuring absorbance at 405 nm (reference: 492 nm) with a microplate reader.

2.4. Measurement of $[Ca^{2+}]_i$

K562 cells were collected by centrifugation (1200–1400 rpm, 3 min) and incubated (10^6 cells/ml) for 30–40 min in a calcium assay buffer (137.5 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.5 mM glucose, 0.6 mM $NaHCO_3$, 20 mM HEPES pH 7.2–7.4 adjusted by NaOH, 0.1% (w/v) bovine serum albumin) in the presence of fluo-3/acetoxymethyl ester form (1 μg/ml). It should be noted that because some of the DIF analogues affect fura-2 fluorometry, we used fluo-3 as a calcium indicator in this study. The fluo-3-loaded cells were washed with the assay buffer two to three times by centrifugation and suspended in the same buffer (10^6 cells/ml).

Fluo-3 fluorometry was carried out at 30°C in a cuvette (2 ml cell suspension per cuvette) using a fluorescence spectrophotometer (F4010, Hitachi, Japan). The fluorescence intensity (emission at 530 nm excited by 490 nm light) of fluo-3 was monitored. The $[Ca^{2+}]_i$ was calculated according to the formula:

$$[Ca^{2+}]_i = K_d \frac{F - F_{min}}{F_{max} - F},$$

where F is fluorescence (at 530 nm) intensity; F_{max} and F_{min} are the fluorescence intensities obtained by adding 0.2% (w/v) Triton X-100 (F_{max}) followed by the addition of 10 mM EGTA (F_{min}); and K_d is the dissociation constant of fluo-3 (400 nM).

3. Results

3.1. Effects of DIF-1 on cell growth and cell differentiation

K562 cells, derived from a patient with chronic myelogenous leukemia (Lozzio and Lozzio, 1975), can be induced to differentiate into erythroid cells or macrophages under appropriate conditions (Sutherland et al., 1986). We have previously shown that DIF-1 suppresses cell growth and induces erythroid differentiation in this cell line (Asahi et al., 1995). We first examined and confirmed the effects

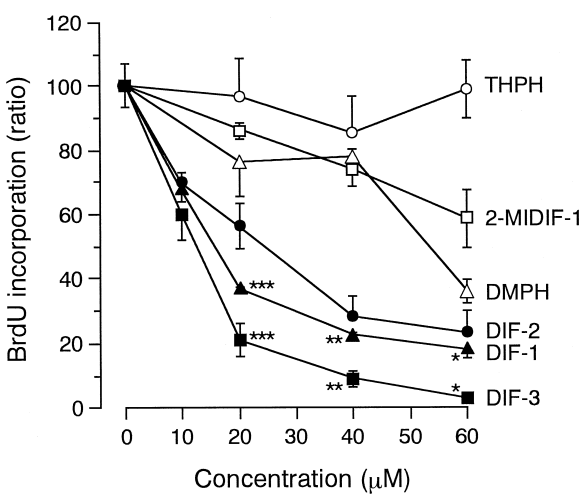

Fig. 4. Effects of DIF analogues on DNA synthesis in K562 cells. K562 cells were incubated in 96-well pates (4×10^4 cells/well) with various concentrations of DIF analogues for 2 h. BrdU was then added to each well and incubated for 2 h. The cells were assayed for BrdU incorporation as described under Section 2. Data are given with the mean values ± S.D. ($n = 4$; 4-well determination was performed) of relative absorbance at 405 nm (reference at 492 nm). $^*P < 0.001$; $^{**}P < 0.005$; $^{***}P < 0.02$ (by Student's t-test).

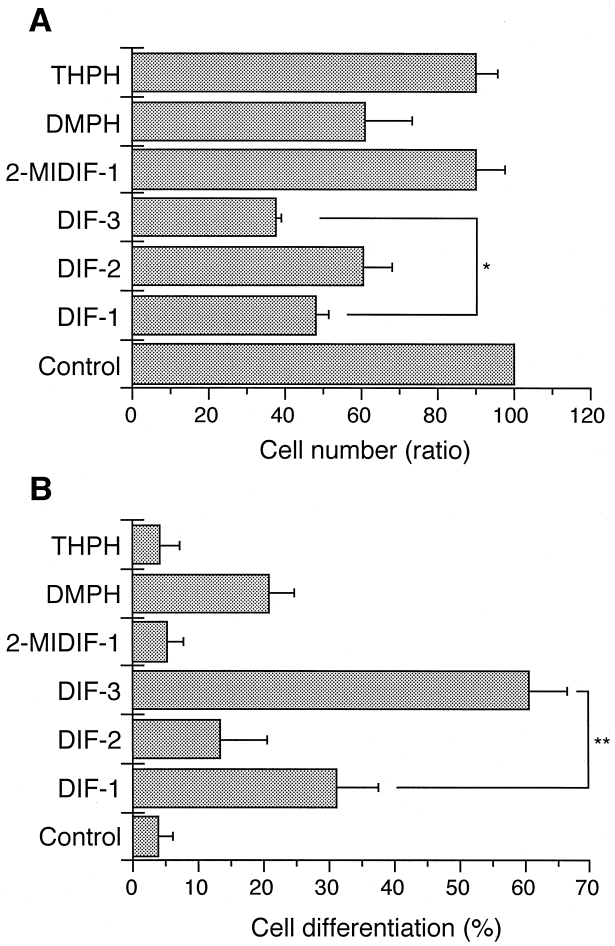

Fig. 5. Effects of DIF analogues on cell growth and erythroid differentiation in K562 cells. K562 cells were incubated at 5×10^4 cells/ml without (control) or with 20 μM DIF analogues. On Day 3, the relative cell number was optically measured with Alamar Blue as described under Section 2 (A). On Day 5, cells were stained with diaminobenzidine, and the stained cells were counted microscopically (B). Data are given with the means ± S.D. of three independent experiments ($n=3$; A) and of six independent experiments ($n=6$; B). $^*P<0.05$; $^{**}P<0.001$ (by Student's *t*-test).

of DIF-1 concentrations on cell growth and cell differentiation in human leukemia K562 cells (Fig. 3).

DIF-1 at 5–30 μM suppressed cell growth and induced erythroid differentiation in a dose-dependent manner (Fig. 3). The results on erythroid differentiation were almost consistent with the previous results, but DIF-1 suppressed cell growth more strongly in this study than in our previous one (Asahi et al., 1995).

3.2. Effects of DIF analogues on DNA synthesis, cell growth and cell differentiation

In order to clarify the chemical structure–effect relation of DIF-like molecules, we examined the effects of six DIF analogues (Fig. 1) on DNA synthesis, cell growth, and cell differentiation in K562 cells. DNA synthesis was inhibited dose-dependently by the analogues, and the order of potency for causing the inhibition was roughly DIF-3 > DIF-1 > DIF-2 > DMPH > 2-MIDIF-1 > THPH (Fig. 4). DIF-3 at 40–60 μM inhibited BrdU incorporation almost completely, but THPH at up to 60 μM did not affect it (Fig. 4).

The effects of the DIF analogues on cell growth and erythroid differentiation were further examined (Fig. 5). DIF-2 at 20 μM suppressed cell growth and induced erythroid differentiation, but its effects were weaker than those of DIF-1. By contrast, at the same concentration, DIF-3 suppressed cell growth and induced erythroid differentiation very strongly. DMPH was almost as effective as DIF-2. Neither 2-MIDIF-1 nor THPH showed marked effects on either cell growth or erythroid differentiation. Since dead cells were not detected on Day 3, the reduction in cell growth by the analogues was not due to cell loss as a result of the toxicity of the analogues (data not shown).

These results indicate that the actions of a DIF-like molecule require a chlorine atom on the 3rd carbon and a methoxy group on the 4th carbon in the benzene ring.

3.3. Effects of DIF analogues on $[Ca^{2+}]_i$

It has been suggested that DIF-1 may function by increasing $[Ca^{2+}]_i$ in higher eukaryotic cells (Kubohara, 1997; Kubohara et al., 1995a,b). We thus examined and compared the effects of DIF analogues on $[Ca^{2+}]_i$ in K562 cells (Fig. 6). As expected, the DIF analogues increased $[Ca^{2+}]_i$ in K562 cells. DIF-3 was most potent, increasing $[Ca^{2+}]_i$ from basal levels (70–100 nM) up to approximately 150 nM (Fig. 6).

3.4. Effects of DIF-3 on cell growth and cell differentiation

We further examined the effects of DIF-3 concentrations on K562 cells (Fig. 7). DIF-3 at 5–30 μM sup-

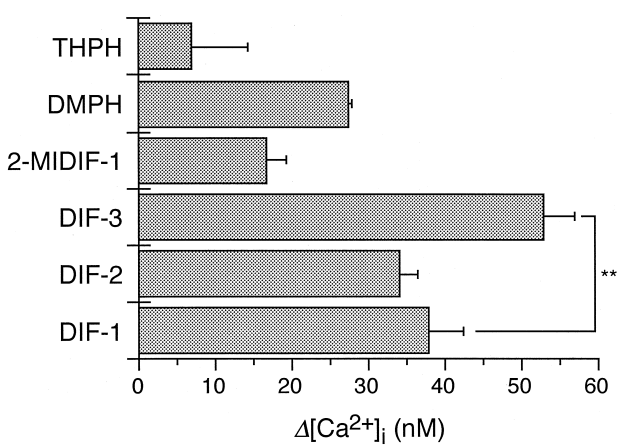

Fig. 6. Effects of DIF analogues on $[Ca^{2+}]_i$ in K562 cells. Fluo-3-loaded K562 cells were stimulated with 20 μM of DIF analogues, and fluorescence intensity was monitored for 4–5 min. Changes in $[Ca^{2+}]_i$ were calculated as described under Section 2. Data are given with the means ± S.D. of three independent experiments ($n=3$). $^{**}P<0.05$ (by Student's *t*-test).

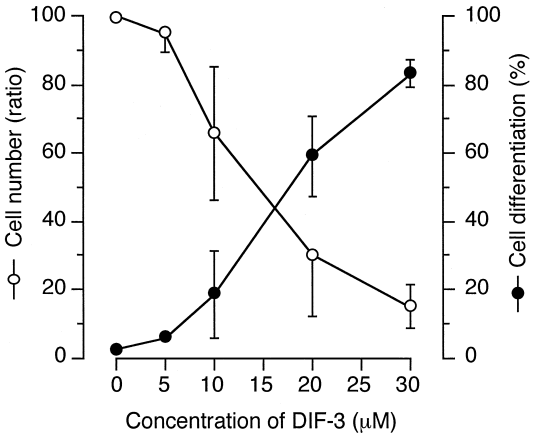

Fig. 7. Effects of DIF-3 on cell growth and erythroid differentiation in K562 cells. K562 cells were incubated at 5×10^4 cells/ml without or with 5–30 μM DIF-3. On Day 3, the relative cell number was optically measured with Alamar Blue as described under Section 2. On Day 5, cells were stained with diaminobenzidine, and the stained cells were counted microscopically. Data are given with the means ± S.D. of three independent experiments ($n = 3$).

pressed cell growth and induced erythroid differentiation in a dose-dependent manner. DIF-3 at 30 μM markedly suppressed cell growth and induced erythroid differentiation by 80–90% (Fig. 7). These results confirm that DIF-3 has greater anti-leukemic activity than DIF-1 (Fig. 3).

4. Discussion

It has been shown previously that the stalk-DIF (DIF-1) of *D. discoideum* suppresses cell growth and induces erythroid differentiation in murine and human leukemia (B8 and K562) cells (Asahi et al., 1995) and also that DIF-1 increases $[Ca^{2+}]_i$, suppressing cell growth or inducing apoptosis in some tumor cells (Kubohara, 1997; Kubohara et al., 1995a,b). It has recently been shown that DIF-1 may be less toxic to normal mammalian cells than to some tumor cells (Kubohara et al., 1998). Thus, it is expected that DIF-1 or its analogues can be applied in chemoprevention/differentiation therapy against cancer and/or may be used as tools for cell science.

In this study, we examined the effects of six DIF analogues (Fig. 1) on the human leukemia K562 cells. We found that the chemical structure of the analogues affected their ability to suppress cell growth and erythroid differentiation, and to increase $[Ca^{2+}]_i$ (Figs. 4–6) and that DIF-3 was the most potent anti-leukemic agent (Figs. 4–7). More precisely, it was shown that one chlorine atom in DIF-3 was required for anti-leukemic activity (growth inhibition and differentiation induction), since DIF-1 (containing two chlorine atoms) and DMPH (no chlorine) were less effective on DNA synthesis, cell growth, and differentiation than DIF-3 (Figs. 4, 5). Both the presence and the location of the methoxy group in the alkylphenone appeared to be of great importance for anti-leukemic activity, since 2-MIDIF-1 (2-methoxy isomer of DIF-1) and THPH (no methoxy group) showed no marked effects on cell growth and erythroid differentiation (Fig. 5) and only a weak effect on DNA synthesis (Fig. 4) and $[Ca^{2+}]_i$ (Fig. 6). Furthermore, since DIF-2 was less effective than DIF-1 (Figs. 4–6), hexanone (the alkyl group in DIF-1) rather than pentanone (in DIF-2) appeared to be important for anti-leukemic activity. Other types of alkyl group should be examined in the future. Although the fine mechanism of the actions of DIFs in tumor cells remains to be elucidated, there seems to be a connection between the anti-leukemic activity of DIF analogues (Figs. 4, 5) and the increase in $[Ca^{2+}]_i$ that these analogues cause (Fig. 6).

It is of importance to note here that DIF-3 was the most potent anti-leukemic agent among the analogues used in this study, in spite of the fact that DIF-1 rather than DIF-3 is the most potent stalk-inducing factor in *Dictyostelium* (Morris et al., 1987; Masento et al., 1988) (Fig. 2). The reason for this difference is presently unknown because the precise mechanisms of action of DIF analogues and even the targets of DIF analogues are unknown both in *Dictyostelium* and in tumor cells. If similar mechanisms are at work in *Dictyostelium* and tumor cells, i.e., if the target molecules of DIF analogues are common in the different cells, the difference in the activity of DIF analogues observed in *Dictyostelium* and K562 cells may reflect evolutional similarity and distinction of the target molecules. In other words, mammals may have ligands similar to DIF-3 that can suppress tumor growth in vivo. However, future studies may yield quite different results.

At any rate, it will be necessary to examine the in vitro and in vivo effects of many other DIF analogues on many other tumor cells in order to develop more potent anti-tumor agents in the near future. The present study should be of value as a reference for future studies.

Acknowledgements

The author thanks Dr. R.R. Kay (MRC Laboratory of Molecular Biology, Cambridge, UK) for supplying some of DIF analogues. This work was supported, in part, by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

References

Asahi, K., Sakurai, A., Takahashi, N., Kubohara, Y., Okamoto, K., Tanaka, Y., 1995. DIF-1, morphogen of *Dictyostelium discoideum*, induces the erythroid differentiation in murine and human leukemia cells. Biochem. Biophys. Res. Commun. 208, 1036–1039.

Azhar, M., Kennady, P.K., Pande, G., Nanjundiah, V., 1997. Stimulation by DIF causes an increase of intracellular Ca^{2+} in *Dictyostelium discoideum*. Exp. Cell Res. 230, 403–406.

Bonner, J.T., 1970. Induction of stalk cell differentiation by cyclic AMP

in the cellular slime mold *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. U.S.A. 65, 110–113.

Kay, R.R., Berks, M., Traynor, D., 1989. Morphogen hunting in *Dictyostelium discoideum*. Development, 81–90, Suppl.

Kubohara, Y., 1997. DIF-1, putative morphogen of *D. discoideum*, suppresses cell growth and promotes retinoic acid-induced cell differentiation in HL-60. Biochem. Biophys. Res. Commun. 236, 418–422.

Kubohara, Y., Okamoto, K., 1994. Cytoplasmic Ca^{2+} and H^{+} concentrations determine cell fate in *Dictyostelium discoideum*. FASEB J. 8, 869–874.

Kubohara, Y., Kimura, C., Tatemoto, K., 1995a. Putative morphogen, DIF, of *Dictyostelium discoideum* induces apoptosis in rat pancreatic AR42J cells. Dev. Growth Differ. 37, 711–716.

Kubohara, Y., Saito, Y., Tatemoto, K., 1995b. Differentiation-inducing factor of *D. discoideum* raises intracellular calcium concentration and suppresses cell growth in rat pancreatic AR42J cells. FEBS Lett. 359, 119–122.

Kubohara, Y., Kabeya, K., Matsui, H., Ishikawa, K., 1998. Effects of DIF-1, an anti-tumor agent isolated from *Dictyostelium discoideum*, on rat gastric mucosal RGM-1 and leptomeningeal cells. Zool. Sci. 15, 713–721.

Lozzio, C.B., Lozzio, B.B., 1975. Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. Blood 45, 321–334.

Masento, M.S., Morris, H.R., Taylor, G.W., Johnson, S.J., Skapski, A.C., Kay, R.R., 1988. Differentiation-inducing factor from the slime mould *Dictyostelium discoideum* and its analogues. Biochem. J. 256, 23–28.

Morandini, P., Offer, J., Traynor, D., Nayler, O., Neuhaus, D., Taylor, G.W., Kay, R.R., 1995. The proximal pathway of metabolism of the chlorinated signal molecule differentiation-inducing factor-1 (DIF-1) in the cellular slime mould *Dictyostelium*. Biochem. J. 306, 735–743.

Morris, H.R., Taylor, G.W., Masento, M.S., Jermyn, K.A., Kay, R.R., 1987. Chemical structure of the morphogen differentiation inducing factor from *Dictyostelium discoideum*. Nature 328, 811–814.

Morris, H.R., Masento, M.S., Taylor, G.W., Jermyn, K.A., Kay, R.R., 1988. Structure elucidation of two differentiation inducing factors (DIF-2 and DIF-3) from the cellular slime mould *Dictyostelium discoideum*. Biochem. J. 249, 903–906.

Schaap, P., Nebl, T., Fisher, P.R., 1996. A slow sustained increase in cytosolic Ca^{2+} levels mediates stalk gene induction by differentiation inducing factor in *Dictyostelium*. EMBO J. 15, 5177–5183.

Sutherland, J.A., Turner, A.R., Mannoni, P., McGann, L.E., Turc, J.M., 1986. Differentiation of K562 leukemia cells along erythroid, macrophage, and megakaryocyte lineages. J. Biol. Response Med. 5, 250–262.

Town, C.D., Gross, J.D., Kay, R.R., 1976. Cell differentiation without morphogenesis in *Dictyostelium discoideum*. Nature 262, 717–719.

Wurster, B., Kay, R.R., 1990. New roles for DIF? Effects on early development in *Dictyostelium*. Dev. Biol. 140, 189–195.