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Structural requirements of *Dictyostelium* differentiation-inducing factors for their stalk-cell-inducing activity in *Dictyostelium* cells and anti-proliferative activity in K562 human leukemic cells

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Abstract

The differentiation-inducing factor-1 (DIF-1) is a lipophilic signal molecule (chlorinated alkylphenone) that induces stalk-cell differentiation in the cellular slime mould *Dictyostelium discoideum*. It has also been shown that DIF-1 and its derivative (DIF-3) suppress cell growth in mammalian tumor cells. In the present study, in order to assess the chemical structure–effect relationship of DIF derivatives and to develop useful agents for the study of both *Dictyostelium* development and cancer biology, we synthesized 28 analogues of DIF-1 and DIF-3 and investigated their stalk-cell-inducing activity in *Dictyostelium* HM44 cells (mutant strain) and anti-proliferative activity in human leukemia K562 cells. HM44 cells are defective in endogenous DIF-1 production and should be suitable for the assay for stalk-cell-inducing activity of DIF analogues. DIF-1 and some of its derivatives at nanomolar levels were good stalk-cell inducers in HM44 cells, whereas DIF-3 and some DIF-3 derivatives at micromolar levels were potent anti-proliferative agents in K562 cells. We also tried to search for antagonistic molecules against DIF-1 and DIF-3 but failed to find such molecules from the analogues used here. The present findings would give us hints for identifying the target molecule(s) of DIFs and also for developing novel anti-cancer drugs.

Keywords: *Dictyostelium*; K562; DIF-1; DIF-3; Stalk-cell differentiation; Anti-cancer drug

1. Introduction

The cellular slime mould *Dictyostelium discoideum* is thought to be an excellent model organism for the study of cell and developmental biology because of its simple pattern of development. Vegetative cells of *D. discoideum* grow as single amoebae by eating bacteria, but when starved they start a developmental program of morphogenesis and gather to form a fruiting body consisting of spores and a multi-cellular stalk at the end of its development. Cyclic AMP (cAMP) is a well-known extra-cellular signal molecule essential for *Dictyostelium* development [1–3]. The differentiation-inducing factors (DIFs) were originally identified as signal molecules that induce stalk-cell differentiation in vitro in the presence of cAMP [4–6]. The most active species, DIF-1 (Scheme 1), has been identified as 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one [5] and DIF-2 (designated DIF-1(−1) in this study; Scheme 1), possessing ~40% of the activity of DIF-1 as 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)pentan-1-one [7,8]. DIF-3 [1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one] (Scheme 1) is the initial product in the process of DIF-1 breakdown and is much less active in inducing stalk-cell differentiation [6,8,9]. DIF-1 is thought to function, at least in part, via an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) [10–12], but the precise signaling system of DIF-1, including the target molecule(s) of DIF-1, is still unknown.

On the other hand, it has been shown that DIFs exhibit strong anti-proliferative activities and occasionally induce cell differentiation in mammalian cells [13–21] and that

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; DIF-1, differentiation-inducing factor-1; DIF-3, differentiation-inducing factor-3

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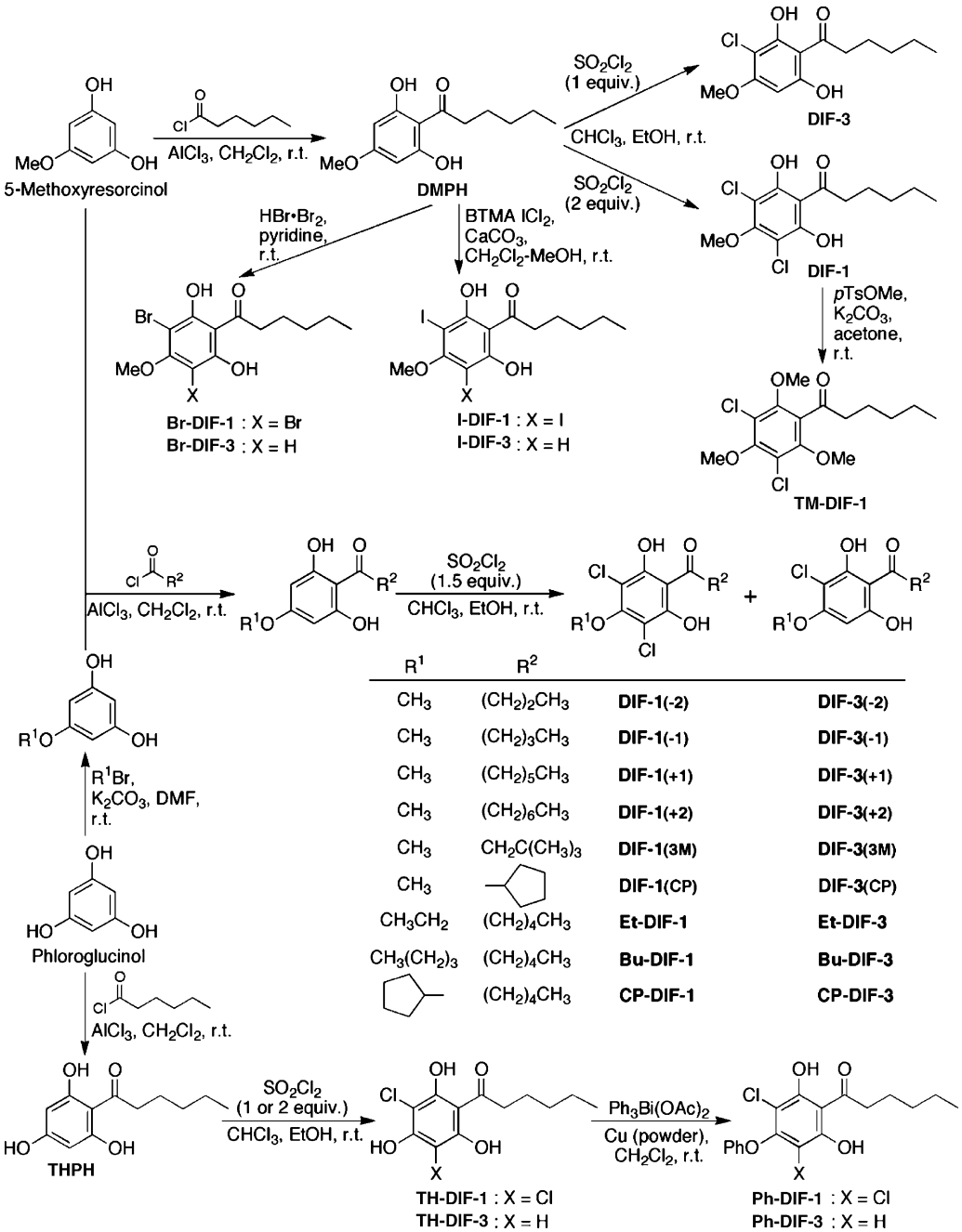

Scheme 1. Synthetic routes to DIF analogues. The DIF analogues were synthesized as described under Section 2.

DIF-3 is a most potent anti-tumor agent among DIFs [17,21]. As to the mechanism of the actions of DIFs, it has been shown that: (1) DIFs increase $[Ca^{2+}]_i$ in some tumor cells [14–17], (2) DIFs activate Akt/protein kinase B (PKB) in human leukemia K562 cells [18], (3) DIF-1 inactivates STAT3 in gastric cancer cells [20], and (4) DIF-1 inhibits the expression of cyclins D/E and the phosphorylation of retinoblastoma protein (pRb) in vascular smooth muscle cells [19] and K562 cells [22]. Quite recently, we have found that calmodulin (CaM)-dependent cyclic nucleotide phosphodiesterase (PDE1) is a pharmacological and specific target of DIFs [23]. Yet, the mechanisms underlying the actions of DIFs in mammalian cells remain to be elucidated.

In the present study, in order to assess the chemical structure–effect relationship of DIF molecules and to develop useful agents for the study of both *Dictyostelium* development and cancer biology, we have synthesized many DIF analogues and examined their stalk-cell-inducing activity in *Dictyostelium* cells and anti-proliferative activity in K562 leukemia cells. In addition, a search for DIF antagonists was also carried out. Here, we have found some potent anti-leukemic agents that possess little stalk-inducing activity in *Dictyostelium*. In this regard, the

chemical structure–effect relationship of the DIF analogues will be discussed.

2. Experimental procedures

2.1. Synthesis of DIF analogues (Scheme 1)

Synthesis of DMPH: $AlCl_3$ (5.46 g and 41.0 mmol) was added to a solution of 5-methoxyresorcinol (2.87 g and 20.5 mmol) in CH_2Cl_2 (40 ml) at room temperature. After 15 min, hexanoyl chloride (4.30 ml and 30.7 mmol) was added. The mixture was stirred for 3 h, poured into water (50 ml), and extracted with ethyl acetate (50 ml) three times. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and evaporated. The residue was chromatographed over silica gel to give DMPH [2.82 g and 11.8 mmol (yield 58%)].

Synthesis of DIF-1 and DIF-3: SO_2Cl_2 (64.0 mg and 0.474 mmol) and EtOH (40 μl) were added to a solution of DMPH (51.4 mg and 0.216 mmol) in $CHCl_3$ (2.5 ml) at room temperature [24]. After being stirred for 1 h, the mixture was evaporated. The residue was chromatographed over silica gel to give DIF-1 [61.6 mg and 0.201 mmol (yield 93%)]. In a similar procedure, DIF-3 was synthesized from DMPH with 1.1 equiv. of SO_2Cl_2.

Synthesis of Br-DIF-1 and Br-DIF-3: Pyridinium tribromide (350 mg and 1.10 mmol) was added to a solution of DMPH (145 mg and 0.609 mmol) in pyridine (4 ml) at room temperature. The mixture was stirred for 1 h, and poured into water (10 ml). Typical aqueous workup and chromatography on silica gel gave Br-DIF-1 [149 mg and 0.375 mmol (yield 62%)] and Br-DIF-3 [53.8 mg and 0.170 mmol (yield 28%)].

Synthesis of I-DIF-1 and I-DIF-3: Calcium carbonate (145 mg and 1.45 mmol) and benzyltrimethylammonium dichloroiodate (BTMA ICl_2) (154 mg and 0.443 mmol) were added to a solution of DMPH (50.6 mg and 0.212 mmol) in CH_2Cl_2–MeOH (2:1) (3 ml) at room temperature [25]. After being stirred for 2 h, the mixture was poured into water (10 ml). Typical aqueous workup and chromatography on silica gel gave I-DIF-1 [59.2 mg and 0.121 mmol (yield 57%)]. In a similar procedure, I-DIF-3 was synthesized from DMPH with 1.1 equiv. of BTMA ICl_2.

Synthesis of TM-DIF-1: Potassium carbonate (208 mg and 1.50 mmol) and methyl *p*-toluenesulfonate (430 mg and 2.31 mmol) were added to a solution of DIF-1 (70.5 mg and 0.230 mmol) in acetone (10 ml) at room temperature. After being stirred for 16 h, the mixture was filtered through a Celite pad. The filter cake was washed by acetone, and the filtrate was concentrated. The residue was chromatographed over silica gel to give TM-DIF-1 [73.2 mg and 0.218 mmol (yield 95%)].

Synthesis of 5-alkoxyresorcinols: Ethyl bromide (1.55 ml and 20.8 mmol) and potassium carbonate (11.0 g and 79.4 mmol) were added to a solution of phloroglucinol (2.01 g and 12.4 mmol) in DMF (64 ml) at room temperature. After being stirred for 8 h, the mixture was poured into 0.1 M hydrochloric acid (250 ml). Typical aqueous workup and chromatography on silica gel gave 5-ethoxyresorcinol [663 mg and 4.30 mmol (yield 35%)]. In a similar procedure, 5-butyloxyresorcinol (yield 29%) and 5-cyclopentyloxyresorcinol (yield 23%) were synthesized from phloroglucinol with *n*-butyl bromide and bromocyclopentane, respectively.

Synthesis of acyl or alkyl group-modified DIF analogues: DIF-1 and DIF-3(−2, −1, +1, +2, 3 M and CP), and TH-, Et-, Bu-, and CP-DIF-1 and DIF-3 were synthesized by the same method of synthesis of DIF-1 and DIF-3. Friedel–Crafts acylation of phloroglucinol, or 5-alkoxyresorcinol with several acyl chloride afforded the corresponding ketones (yield 35–75%). Chlorination of these ketones with 1.5 equiv. of SO_2Cl_2 gave corresponding DIF-1 and DIF-3 analogues (yield 17–45%, each).

Synthesis of Ph-DIF-1 and Ph-DIF-3: Triphenylbismuth diacetate ($Ph_3Bi(OAc)_2$) (71.3 mg and 0.128 mmol) and copper powder (1.4 mg and 0.022 mmol) were added to a solution of TH-DIF-1 (36.9 mg and 0.126 mmol) in CH_2Cl_2 (2 ml) at room temperature [26,27]. After being stirred for 2 h, the mixture was filtered through a Celite pad. The filter cake was washed by ethyl acetate and the filtrate was concentrated. The residue was chromatographed to give Ph-DIF-1 [19.2 mg and 0.052 mmol (yield 41%)]. In a similar procedure, Ph-DIF-3 was synthesized from TH-DIF-3.

2.2. Data for synthetic DIF analogues

DIF-1: Yellow amorphous solid; ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.38 (2H, br.s), 3.97 (3H, s), 3.12 (2H, t, J = 7.3 Hz), 1.68–1.73 (2H, m), 1.34–1.37 (4H, m), 0.91 (3H, t, J = 7.0 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 206.6, 157.4, 156.3 (2C), 107.4 (2C), 107.1, 61.0, 44.4, 31.4, 23.9, 22.5, 13.9; HREIMS m/z 306.0414 $[M]^+$ (306.0426 calculated for $C_{13}H_{16}O_4Cl_2$).

DIF-3: Colorless amorphous solid; ^{1}H NMR (600 MHz, acetone-d_6) δ 6.22 (1H, s), 3.89 (3H, s), 3.10 (2H, t, J = 7.7 Hz), 1.64–1.69 (2H, m), 1.32–1.34 (4H, m), 0.89 (3H, t, J = 7.0 Hz); ^{13}C NMR (150 MHz, acetone-d_6) δ 207.2, 162.6, 161.6, 160.3, 105.9, 100.8, 92.5, 56.7, 44.6, 32.3, 25.0, 23.2, 14.2; HREIMS m/z 272.0823 $[M]^+$ (272.0815 calculated for $C_{13}H_{17}O_4Cl$).

Br-DIF-1: Yellow amorphous solid; ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.45 (2H, br.s), 3.93 (3H, s), 3.13 (2H, t, J = 7.4 Hz), 1.68–1.72 (2H, m), 1.33–1.37 (4H, m), 0.91 (3H, t, J = 7.0 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 206.6, 159.3, 157.9 (2C), 107.4, 97.8 (2C), 60.8, 44.4, 31.4, 23.9, 22.5, 13.9; HREIMS m/z 393.9428 $[M]^+$ (393.9415 calculated for $C_{13}H_{16}O_4Br_2$).

Br-DIF-3: Colorless amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.66 (1H, br.s), 6.80 (1H, br.s), 6.12 (1H, s), 3.89 (3H, s), 3.08 (2H, t, J = 7.5 Hz),

1.66–1.73 (2H, m), 1.33–1.38 (4H, m), 0.91 (3H, t, $J = 7.0$ Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 206.0, 165.6, 160.9, 155.1, 104.8, 93.5, 90.5, 56.5, 44.0, 31.5, 24.2, 22.5, 14.0; HREIMS *m/z* 316.0296 $[M]^+$ (316.0310 calculated for $C_{13}H_{17}O_4Br$).

I-DIF-1: Yellow amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.48 (2H, br.s), 3.89 (3H, s), 3.13 (2H, t, $J = 7.3$ Hz), 1.67–1.72 (2H, m), 1.33–1.36 (4H, m), 0.91 (3H, t, $J = 6.5$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.3, 163.8, 161.4 (2C), 106.6, 73.1 (2C), 60.7, 44.2, 31.5, 24.1, 22.6, 14.1; HREIMS *m/z* 489.9142 $[M]^+$ (489.9138 calculated for $C_{13}H_{16}O_4I_2$).

I-DIF-3: Yellow amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.32 (1H, br.s), 7.15 (1H, br), 6.08 (1H, s), 3.88 (3H, s), 3.08 (2H, t, $J = 7.5$ Hz), 1.67–1.71 (2H, m), 1.33–1.37 (4H, m), 0.91 (3H, t, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.0, 166.6, 162.8, 157.9, 104.7, 93.1, 67.5, 56.7, 44.0, 31.6, 24.3, 22.6, 14.1; HREIMS *m/z* 364.0163 $[M]^+$ (364.0172 calculated for $C_{13}H_{17}O_4I$).

TM-DIF-1: Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.84 (3H, s), 3.74 (6H, s), 2.68 (2H, t, $J = 7.3$ Hz), 1.58–1.62 (2H, m), 1.24–1.27 (4H, m), 0.82 (3H, t, $J = 6.6$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.3, 154.2, 151.7 (2C), 129.1, 119.5 (2C), 62.6 (2C), 60.8, 44.9, 31.2, 23.0, 22.4, 13.9; HREIMS *m/z* 334.0726 $[M]^+$ (334.0739 calculated for $C_{15}H_{20}O_4Cl_2$).

DIF-1(−2): Yellow amorphous solid; HREIMS *m/z* 278.0088 $[M]^+$ (278.0113 calculated for $C_{11}H_{12}O_4Cl_2$).

DIF-3(−2): Colorless amorphous solid; HREIMS *m/z* 244.0475 $[M]^+$ (244.0502 calculated for $C_{11}H_{13}O_4Cl$).

DIF-1(−1): Yellow amorphous solid; HREIMS *m/z* 292.0259 $[M]^+$ (292.0269 calculated for $C_{12}H_{14}O_4Cl_2$).

DIF-3(−1): Colorless amorphous solid; HREIMS *m/z* 258.0652 $[M]^+$ (258.0659 calculated for $C_{12}H_{15}O_4Cl$).

DIF-1(+1): Yellow amorphous solid; HREIMS *m/z* 320.0581 $[M]^+$ (320.0582 calculated for $C_{14}H_{18}O_4Cl_2$).

DIF-3(+1): Colorless amorphous solid; HREIMS *m/z* 286.0961 $[M]^+$ (286.0972 calculated for $C_{14}H_{19}O_4Cl$).

DIF-1(+2): Yellow amorphous solid; HREIMS *m/z* 334.0719 $[M]^+$ (334.0739 calculated for $C_{15}H_{20}O_4Cl_2$).

DIF-3(+2): Colorless amorphous solid; HREIMS *m/z* 300.1121 $[M]^+$ (300.1128 calculated for $C_{15}H_{21}O_4Cl$).

DIF-1(*3M*): Yellow amorphous solid; HREIMS *m/z* 306.0392 $[M]^+$ (306.0426 calculated for $C_{13}H_{16}O_4Cl_2$).

DIF-3(*3M*): Yellow amorphous solid; HREIMS *m/z* 272.0800 $[M]^+$ (272.08715 calculated for $C_{13}H_{17}O_4Cl$).

DIF-1(*CP*): Yellow amorphous solid; HREIMS *m/z* 304.0262 $[M]^+$ (304.0269 calculated for $C_{13}H_{14}O_4Cl_2$).

DIF-3(*CP*): Colorless amorphous solid; HREIMS *m/z* 270.0657 $[M]^+$ (270.0659 calculated for $C_{13}H_{15}O_4Cl$).

THPH: Yellow amorphous solid; HREIMS *m/z* 224.1028 $[M]^+$ (224.1049 calculated for $C_{12}H_{16}O_4$).

TH-DIF-1: Yellow amorphous solid; HREIMS *m/z* 292.0244 $[M]^+$ (292.0269 calculated for $C_{12}H_{14}O_4Cl_2$).

TH-DIF-3: Yellow amorphous solid; HREIMS *m/z* 258.0641 $[M]^+$ (258.0659 calculated for $C_{12}H_{15}O_4Cl$).

Ph-DIF-1: Yellow amorphous solid; HREIMS *m/z* 368.0577 $[M]^+$ (368.0582 calculated for $C_{18}H_{18}O_4Cl_2$).

Ph-DIF-3: Colorless amorphous solid; HREIMS *m/z* 334.0957 $[M]^+$ (334.0972 calculated for $C_{18}H_{10}O_4Cl$).

Et-DIF-1: Yellow amorphous solid; HREIMS *m/z* 320.0571 $[M]^+$ (320.0582 calculated for $C_{14}H_{18}O_4Cl_2$).

Et-DIF-3: Colorless amorphous solid; HREIMS *m/z* 286.0948 $[M]^+$ (286.0972 calculated for $C_{14}H_{19}O_4Cl$).

Bu-DIF-1: Yellow amorphous solid; HREIMS *m/z* 348.0894 $[M]^+$ (348.0895 calculated for $C_{16}H_{22}O_4Cl_2$).

Bu-DIF-3: Yellow amorphous solid; HREIMS *m/z* 314.1264 $[M]^+$ (314.1285 calculated for $C_{16}H_{23}O_4Cl$).

CP-DIF-1: Yellow amorphous solid; HREIMS *m/z* 360.0897 $[M]^+$ (360.0895 calcd for $C_{17}H_{22}O_4Cl_2$).

CP-DIF-3: Yellow amorphous solid; HREIMS *m/z* 326.1272 $[M]^+$ (326.1285 calculated for $C_{17}H_{23}O_4Cl$).

2.3. Cells

Dictyostelium DIF-deficient mutant HM44 cells [28] and human leukemia K562 cells were used in this study. K562 cells were maintained at 37 °C (5% CO_2) in tissue culture dishes filled with a growth medium (an RPMI1640 medium with 10% fetal bovine serum, 25 μg/ml penicillin, and 50 μg/ml streptomycin; designated RPMI).

2.4. Assay for stalk-cell-inducing activity of DIF analogues in HM44 cells

HM44 cells were grown in association with *Klebsiella aerogenes* on modified SM agar plates [29,30]. Cells were collected and allowed to differentiate in vitro (at 21 °C) in multi-well (12) plates, each well (2×10^5 cells/well) containing 0.5 ml of a salt solution (5 mM cAMP, 2 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 50 μg/ml penicillin, 100 μg/ml streptomycin sulfate, 10 mM MES-KOH pH 6.2) in the presence of DIF analogues or a vehicle (EtOH). On day 2, cells were observed using a phase-contrast microscope (stalk cells can be identified by their vacuolated morphology), and stalk-cell population (percentage of total cells) was assessed (usually, more than 150 cells were counted per well).

2.5. Assay for anti-proliferative activity of DIF analogues in K562 cells

K562 cells were incubated at 37 °C (5% CO_2) in a multi-well (12) plate, each well containing 1 ml of RPMI (2–3×10^4 cells/ml) in the presence of DIF analogues or vehicle (EtOH). On day 3, 50 μl of Alamar Blue (cell number indicator) was added to each well, and the cells were incubated at 37 °C (5% CO_2) until the color of Alamar Blue changed (usually for 1–2 h). Then, 200 μl of each of the sample solutions was transferred into a 96-well plate, and absorbance at 570 nm (reference at 595 nm) was measured with a micro-plate reader (Bio-Rad, Model 550). A cell number was given as percentage of the control absorbance.

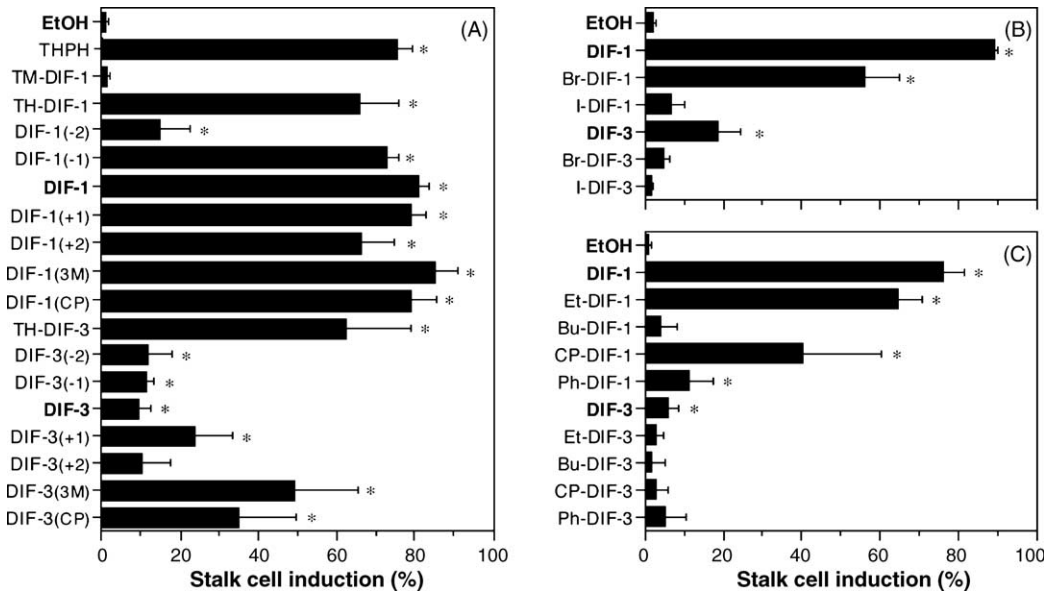

Fig. 1. Effects of DIF analogues on stalk-cell formation in HM44 cells. HM cells were incubated in vitro with a vehicle (0.2% EtOH) or 2 nM of DIF analogues for 2 days, and stalk-cell population (percentage of total cells) was assessed using a phase-contrast microscope (A–C are the results of different series of experiments). Results are presented as the mean values (±S.D.) of three (B; $n = 3$) or four (A and C; $n = 4$) independent experiments. It should be noted that, since 2 nM is nearly a saturated concentration for some DIF-1 derivatives to induce stalk-cell differentiation in this assay system (see Fig. 2), the mean values do not reflect the proper relative activities of the analogues. $^{*}P < 0.05$ vs. control (EtOH) (by Student's t-test).

3. Results

3.1. Synthesis of DIF analogues

DIF analogues were chemically synthesized as described in Section 2 and termed as shown in Scheme 1 for the sake of convenience, although DIF-1(−1) and TH-DIF-1 were originally termed as DIF-2 and dichloro-THPH, respectively [8,31].

3.2. Effects of DIF analogues on stalk-cell differentiation in Dictyostelium HM44 cells

Dictyostelium wild-type strain V12M2 can differentiate into stalk cells in an in vitro monolayer culture in the presence of extra-cellular cAMP, since the cells produce stalk-cell-inducing factors (DIFs). Under the same in vitro conditions, however, HM44 cells cannot differentiate into stalk cells unless DIFs are supplied because this mutant strain is deficient in DIF production [28,30]. Thus, we assayed the stalk-cell-inducing activity of DIF analogues using the in vitro culture of HM44 cells. As expected, DIF-1 was the most potent stalk-cell inducer, and DIF-3 was much less active (Fig. 1). Roughly estimating, the more similar to DIF-1 the analogues were, the stronger the stalk-cell-inducing activities that they showed (Fig. 1). Because THPH and TH-DIF-1 are the precursors of DIF-1 in its biosynthesis pathway [31], stalk-cell-inducing activities of THPH and TH-DIF-1 (Fig. 1A) could be exerted via DIF-1 produced from THPH and TH-DIF-1 by HM44

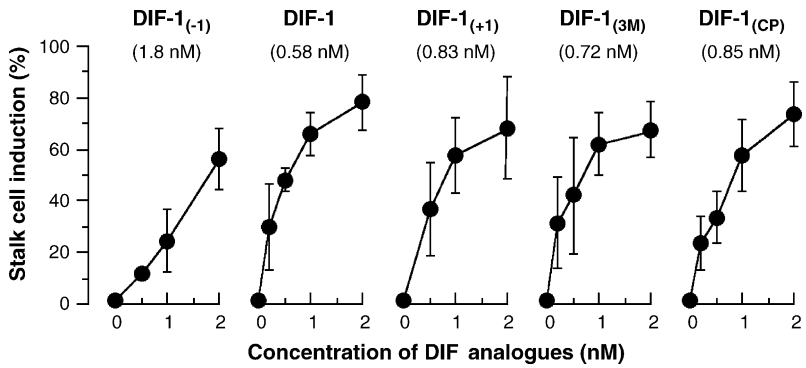

Fig. 2. Detailed analysis for the stalk-cell-inducing activity of DIF-1 derivatives in HM44 cells. HM44 cells were incubated in vitro with a vehicle (0.2% EtOH) or the indicated concentrations of DIF-1 derivatives, and stalk-cell population (percentage of total cells) was assessed using a phase-contrast microscope. Results are presented as the mean values (±S.D.) of three independent experiments ($n = 3$). The concentration required for 50% stalk-cell induction in this assay (ED_{50}) obtained from the graph is shown in parenthesis.

cells, and thus, THPH and TH-DIF-1 themselves may be regarded as inactive analogues. As expected from a previous study using a different assay system [7], DIF-1(−2), DIF-1(−1), DIF-1(+1), and DIF-1(+2) were less effective than DIF-1 (Figs. 1A and 2). Interestingly, 3M-DIF-1 and CP-DIF-1 possessed a potent biological activity with ED_{50} values of ∼0.72 nM and ∼0.85 nM, respectively, whereas the ED_{50} value of DIF-1 was ∼0.58 nM (Fig. 2). Halogen substitution or modification of the methoxy group in DIF-1 greatly reduced the stalk-cell-inducing activity (Fig. 1B and C).

3.3. Search for DIF antagonists in HM44 cells

Antagonists for DIF-1 could be powerful tools for the analysis of the DIF-1-signaling system in *D. discoideum*. Fundamentally, DIF analogues that do not have apparent stalk-cell-inducting activity may include such antagonists, which would inhibit DIF-1-induced stalk-cell formation. To search for antagonists for DIF-1, we examined the effects of TM-DIF-1 and I-DIF-3 on DIF-1-induced stalk-cell formation in HM44 (Fig. 3). However, TM-DIF-1 at 4–80 μM by itself induced stalk-cell formation and did not much inhibited DIF-1-induced stalk-cell formation (Fig. 3A). TM-DIF-1 at less than 4 μM was inactive so that TM-DIF-1 neither induced stalk-cell formation nor inhibited DIF-1-induced stalk-cell formation (data not shown). On the other hand, I-DIF-3 at 0.02–0.5 μM did not show an antagonistic effect toward DIF-1 (Fig. 3B), while I-DIF-3 at more than 0.5 μM was toxic to the cells (data not shown).

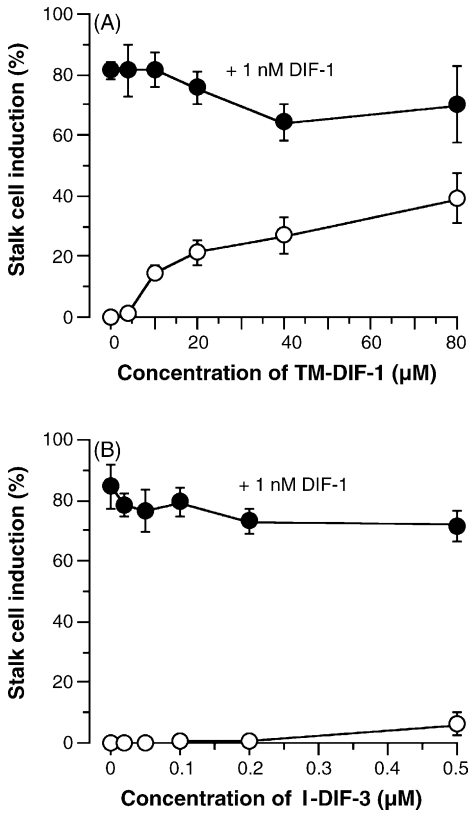

Fig. 3. Search for DIF-1 antagonists in HM44 cells. HM44 cells were incubated in vitro with a vehicle (0.2% EtOH) or the indicated concentrations of TM-DIF-1 (A) or I-DIF-3 (B) in the presence (closed circles) or absence (open circles) of 1 nM DIF-1, and stalk-cell population (percentage of total cells) was assessed using a phase-contrast microscope. Results are presented as the mean values (±S.D.) of three independent experiments ($n = 3$).

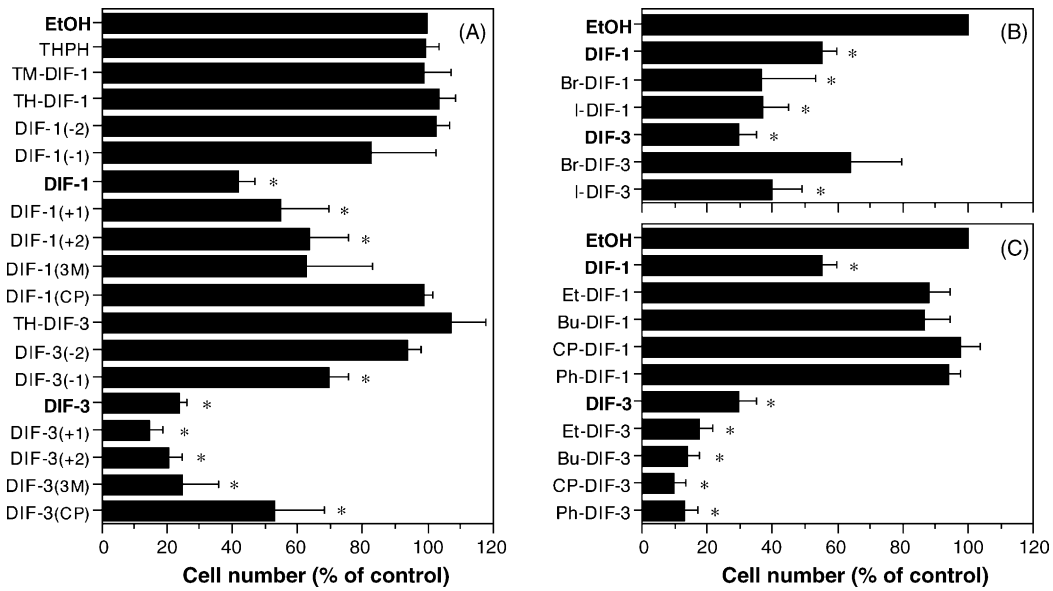

Fig. 4. Effects of DIF analogues on cell growth in K562 cells. K562 cells were incubated in vitro with a vehicle (0.15% EtOH) or 15 μM of DIF analogues for 3 days, and the relative cell number was assessed (A–C are the results of different series of experiments). Results are presented as the mean values (±S.D.) of three independent experiments ($n = 3$). It should be noted that since 15 μM is nearly a saturated concentration for some DIF-3 derivatives to induce stalk-cell differentiation in this assay system (see Fig. 5), the mean values do not reflect the proper relative activities of the analogues. $^{*}P < 0.05$ vs. control (EtOH) (by Student's *t*-test).

3.4. Effects of DIF analogues on cell growth in K562 human leukemia cells

It has been shown that DIFs possess anti-proliferative activity in vitro in some tumor cells [13–17,20,21]. As a first step to obtain more potent and useful anti-cancer agents, we checked the effects of DIF analogues on cell growth in K562 human leukemia cells (Fig. 4). By clear contrast to stalk-cell-inducing activity in HM44 cells (Fig. 1), some derivatives of DIF-3 showed potent anti-proliferative activity in K562 cells (Fig. 4). Of the DIF-3 derivatives with modified alkyl side chain, DIF-3(+1) was most potent (Fig. 5A). Halogen substitution of DIFs showed similar levels of anti-proliferative activity, but DIF-3 was the most potent among them (Fig. 5B). Furthermore, Bu-DIF-3 and CP-DIF-3 were found to possess

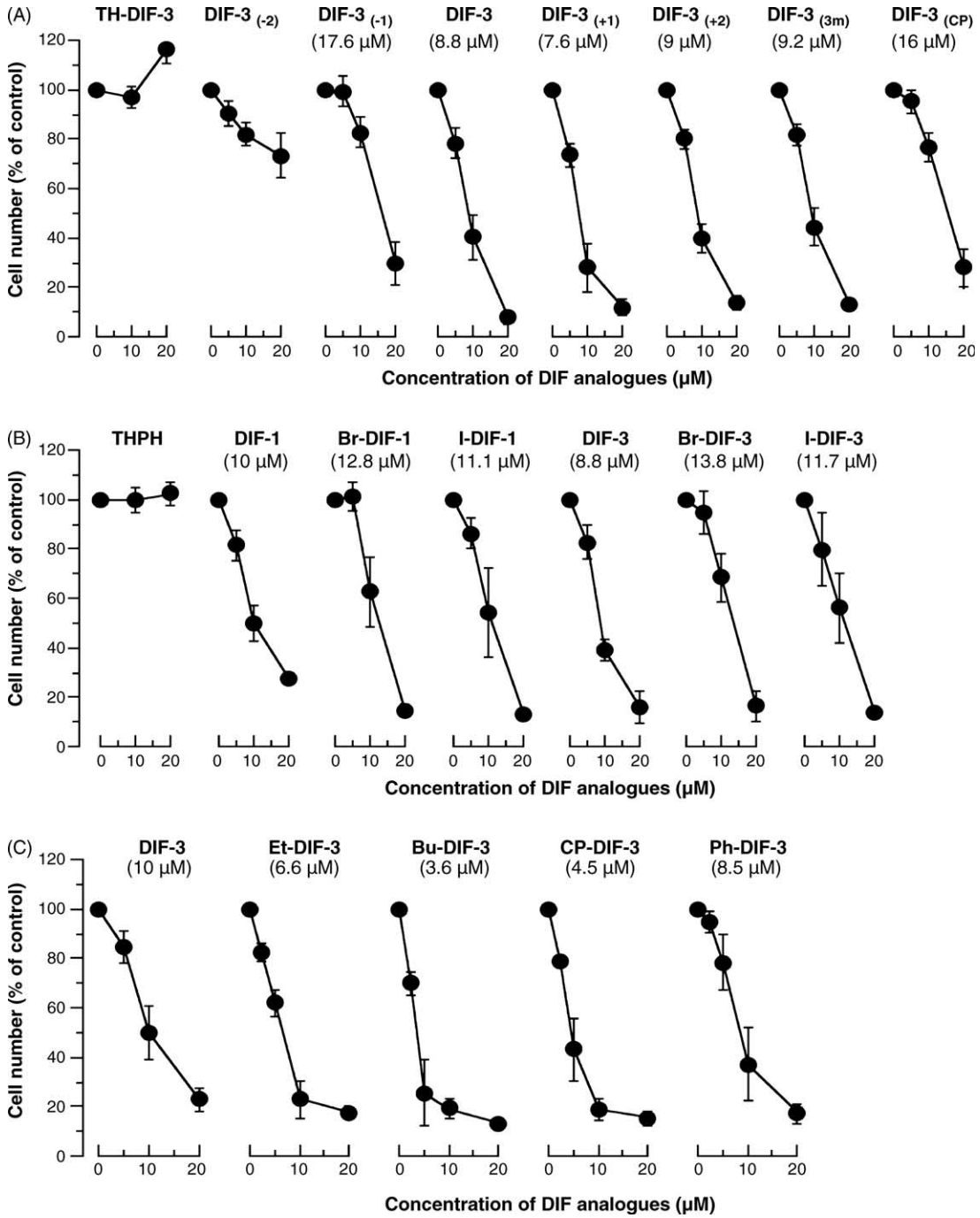

Fig. 5. Detailed analysis for the anti-proliferative activity of DIF-3 derivatives in K562 cells. K562 cells were incubated in vitro with a vehicle (0.2% EtOH) or the indicated concentrations of DIF-3 derivatives for 3 days, and the relative cell number was assessed (A–C are the results of different series of experiments). Results are presented as the mean values (±S.D.) of four independent experiments ($n = 4$). The concentration required for 50% growth inhibition (ID_{50}) in this assay obtained from each graph is shown in parentheses.

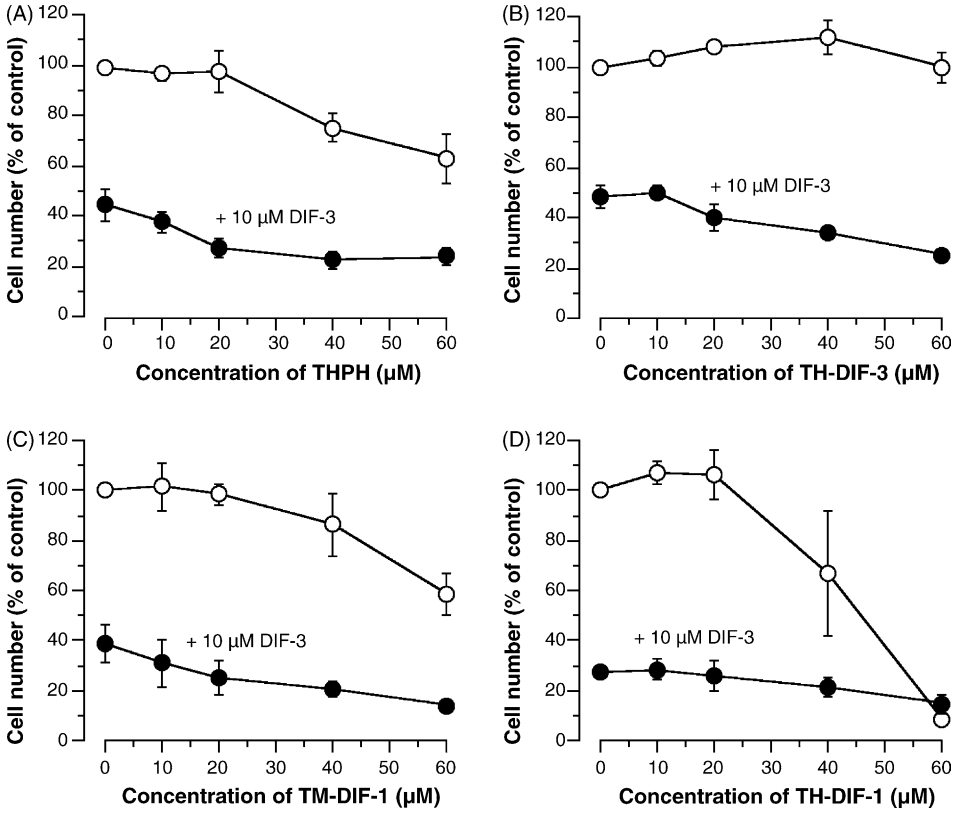

Fig. 6. Search for DIF-3 antagonists in K562 cells. K562 cells were incubated in vitro with a vehicle (0.2% EtOH) or the indicated concentrations of THPH (A), TH-DIF-3 (B), TM-DIF-1 (C), or TH-DIF-1 (D) in the presence (closed circles) or absence (open circles) of 10 μM DIF-3, and relative cell number was assessed on day 3. Results are presented as the mean values (±S.D.) of three independent experiments ($n = 3$).

strong activity with ID_{50} values of ~3.6 μM and ~4.5 μM, respectively, while the ID_{50} value of DIF-3 was ~10 μM in the assay (Fig. 5C).

3.5. Search for DIF antagonists in K562 cells

In order to find DIF antagonists, we examined the effects of candidate molecules (THPH, TH-DIF-3, TM-DIF-1, and TH-DIF-1) on DIF-3-induced growth inhibition in K562 cells (Fig. 6). Unfortunately, however, these molecules at up to 60 μM never recovered the cell growth suppressed by 10 μM DIF-3 and rather showed additive effects with DIF-3, indicating that these are not DIF-3 antagonists in this assay. THPH, TM-DIF-1, and TH-DIF-1 at more than 20 μM suppressed cell growth by themselves.

4. Discussion

In the present study, which examines the effects of DIF analogues on *Dictyostelium* HM44 cells and human leukemia K562 cells, we have assessed the structural requirements of DIF analogues for their biological and pharmacological activities in both cell lines. As shown here, DIF-1 and some of its derivatives are good stalk-cell inducers in HM44 cells, whereas DIF-3 and some DIF-3 derivatives are potent anti-proliferative agents in K562 cells. In addition, we have tried to search for antagonistic molecules against DIFs but failed to find such molecules from the analogues used here (Figs. 3 and 6).

4.1. Stalk-cell-inducing activity of DIF analogues in HM44 cells

The stalk-cell-inducing activity of some DIF-1 analogues, such as DIF-1(−2), DIF-1(−1), DIF-1, DIF-1(+1), DIF-1(+2), and Br-DIF-1, has been assayed before by Massento et al. [7] using a different in vitro assay system with wild-type V12M2 cells at a density of 10^4 cells/ml, while DIF-deficient mutant HM44 cells at 4×10^5 cells/ml were used in our assay. Probably because of the different assay systems (e.g., DIF breakdown activity must be different in the systems), the apparent ED_{50} values of DIF-1, DIF-1(−1), DIF-1(+1), and Br-DIF-1 obtained in both studies would be somewhat different from one another.

Most interestingly, it was found here that DIF-1 (3M) and DIF-1 (CP), rather than DIF-1(−1) and DIF-1(+2), possess considerable levels of stalk-cell-inducing activity (Figs. 1A and 2), suggesting that five carbon molecules at the position of the alkyl side chain should be the best for the analogues to exert biological activity. Furthermore, it is noteworthy that while DIF-1 (CP) possesses bioactivity in HM44 cells, this molecule exhibits no marked activity in

K562 cells (Figs. 1 and 4). Analyses of many other DIF derivatives may give us hints for identifying the receptor(s) of DIF-1 in *D. discoideum* in the future.

4.2. Anti-proliferative activity of DIF analogues in K562 cells

As already mentioned, DIFs possess anti-tumor activities in several cell lines [13–17,20,21], and the order of potency for anti-proliferative activity was established as DIF-3 > DIF-1 > DIF-2 (designated DIF-1(−1) in this study) in K562 cells [17]. Here, we have added interesting profiles of the many newly synthesized DIF analogues. Of the present findings, it is noteworthy that Bu-DIF-3 and CP-DIF-3 possess strong anti-proliferative activity (Fig. 5C). This would be valuable information in the development of more effective and useful derivatives of DIF-3 and indicates that the pharmacological activity of some other derivatives having modifications at the position of the methoxy group in DIF-3 should be examined in the future.

Recently, we reported that PDE1 is a pharmacological and specific target of DIFs [23]. However, some of the known actions of DIFs in mammalian cells do not seem to be exerted via PDE1 inhibition by DIFs. For example, a rise in $[Ca^{2+}]_i$ by DIFs [14,16,17] may not be explained by PDE1 inhibition by them, suggesting that there should be some other target(s) of DIFs in mammalian cells. The present findings would give us hints for identifying the pharmacological target(s) of DIFs in mammalian cells and also for developing novel anti-cancer drugs.

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