RESEARCH ARTICLE

Properties of a non-bioactive fluorescent derivative of differentiation-inducing factor-3, an anti-tumor agent found in Dictyostelium discoideum

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ABSTRACT

Differentiation-inducing factor-3 (DIF-3), found in the cellular slime mold Dictyostelium discoideum, and its derivatives, such as butoxy-DIF-3 (Bu-DIF-3), are potent anti-tumor agents. To investigate the activity of DIF-like molecules in tumor cells, we recently synthesized a green fluorescent DIF-3 derivative, BODIPY-DIF-3G, and analyzed its bioactivity and cellular localization. In this study, we synthesized a red (orange) fluorescent DIF-3 derivative, BODIPY-DIF-3R, and compared the cellular localization and bioactivities of the two BODIPY-DIF-3s in HeLa human cervical cancer cells. Both fluorescent compounds penetrated the extracellular membrane within 0.5 h and localized mainly to the mitochondria. In formalin-fixed cells, the two BODIPY-DIF-3s also localized to the mitochondria, indicating that the BODIPY-DIF-3s were incorporated into mitochondria independently of the mitochondrial membrane potential. After treatment for 3 days, BODIPY-DIF-3G, but not BODIPY-DIF-3R, induced mitochondrial swelling and suppressed cell proliferation. Interestingly, the swollen mitochondria were stainable with BODIPY-DIF-3G but not with BODIPY-DIF-3R. When added to isolated mitochondria in vitro, BODIPY-DIF-3G increased dose-dependently the rate of O₂ consumption, but BODIPY-DIF-3R did not. These results suggest that the bioactive BODIPY-DIF-3G suppresses cell proliferation, at least in part, by altering mitochondrial activity, whereas the non-bioactive BODIPY-DIF-3R localizes to the mitochondria but does not affect mitochondrial activity or cell proliferation.

KEY WORDS: Dictyostelium discoideum, DIF, Anti-tumor agent, Mitochondria, Uncoupler

INTRODUCTION

The cellular slime mold Dictyostelium discoideum (D. discoideum) is a soil microorganism that, at the end of its life cycle, transforms into a multicellular fruiting body consisting of a stalk and spores. Differentiation-inducing factor-1 (DIF-1) (Fig. 1A) is a putative morphogen that regulates cell fate by inducing the differentiation of prestalk cells and suppressing the differentiation of prespore cells (Kay et al., 1989; Kay et al., 1999; Morris et al., 1987). DIF-1 has been shown to function also as a modulator of chemotaxis in D. discoideum (Kuwayama and Kubohara, 2009). Differentiation-inducing factor-3 (DIF-3) (Fig. 1A) is the first metabolite formed during DIF-1 degradation and it has virtually no activity in the induction of prestalk cells and the modulation of chemotaxis (Kay et al., 1989; Kay et al., 1999; Kuwayama and Kubohara, 2009; Morris et al., 1988).

DIF-1 and DIF-3 have been shown to suppress tumor cell proliferation as well as induce and promote cell differentiation in de-differentiated tumor cells (Asahi et al., 1995; Jingushi et al., 2012; Jingushi et al., 2013; Kanai et al., 2003; Kubohara et al., 1995; Kubohara, 1997; Kubohara, 1999; Takahashi-Yanaga et al., 2003; Takahashi-Yanaga et al., 2006). Interestingly, DIF-3 is more active than DIF-1 in suppressing cell proliferation and inducing erythroid differentiation of K562 human myelogenous leukemia cells (Akaishi et al., 2004; Kubohara, 1999). We previously found that chemically modified derivatives of DIF-3 such as butoxy-DIF-3 (Bu-DIF-3), which contains a modification at the methoxy group (Fig. 1A), are more potent anti-proliferative agents than DIF-3 in K562 cells (Gokan et al., 2005). Thus, DIF-3 derivatives may be promising anti-cancer drugs. However, the precise mechanisms underlying the function of DIF-like molecules in mammalian cells remain to be elucidated.

To study the cellular localization, function, and target proteins of DIF-3-like molecules in mammalian cells, we recently synthesized a green fluorescent derivative of DIF-3, BODIPY-DIF-3 (designated BODIPY-DIF-3G in this study) (Fig. 1B), and showed that BODIPY-DIF-3G localizes mainly to the mitochondria in HeLa human cervical cancer cells (Kubohara et al., 2013). We also showed that BODIPY-DIF-3G has similar biological activity as DIF-3 and Bu-DIF-3, suppressing cell proliferation in part, by acting as a mitochondrial uncoupler to disrupt mitochondrial function (Kubohara et al., 2013).

In the present study, we synthesized a red (orange) fluorescent derivative of DIF-3, BODIPY-DIF-3R (Fig. 1B), and showed that BODIPY-DIF-3R also localized mainly to the mitochondria. However, unlike BODIPY-DIF-3G and DIF-3, BODIPY-DIF-3R did not suppress HeLa cell proliferation nor induce any change in mitochondrial morphology or function. These results show that bioactive DIF-like molecules suppress cell proliferation at least in part via disturbance of mitochondrial activity, whereas BODIPY-DIF-3R localizes to the mitochondria but is not bioactive. Our results also indicate that the swollen mitochondria are morphologically, biochemically, and thus functionally different, from normal mitochondria and can be distinguished by staining with BODIPY-DIF-3G and BODIPY-DIF-3R.
RESULTS
Synthesis of fluorescent derivatives of DIF-3 and their effects on HeLa cell proliferation

We previously synthesized a green fluorescent derivative of DIF-3, BODIPY-DIF-3G (Fig. 1B), and elucidated its cellular localization and function in HeLa cells (Kubohara et al., 2013). Here, we synthesized another reagent for analyzing DIF-like molecules, a red (orange) fluorescent derivative of DIF-3, BODIPY-DIF-3R (Fig. 1B).

We first compared the effects of BODIPY-DIF-3G or BODIPY-DIF-3R on HeLa cell proliferation. As described previously (Kubohara et al., 2013), 20 μM of DIF-3 or BODIPY-DIF-3G markedly suppressed cell proliferation compared to dimethyl sulfoxide (DMSO) control. Unexpectedly, 20 μM BODIPY-DIF-3R had very little effect on cell proliferation (Fig. 2A). Cells remained viable and appeared healthy even after a 3-day incubation period with each of the three compounds (Fig. 2B).

Fig. 1. Chemical structures of DIF-1, DIF-3, and Bu-DIF-3, and synthesis of BODIPY-conjugated compounds. (A) DIF-1 [1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one] and DIF-3 [1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one] are endogenous factors in D. discoideum. Bu-DIF-3 (butoxy-DIF-3) [1-(3-chloro-2,6-dihydroxy-4-butoxyphenyl)hexan-1-one] is an artificially designed derivative of DIF-3. The order of anti-proliferative activity has been established as Bu-DIF-3 > DIF-3 > DIF-1 (Gokan et al., 2005). (B) BODIPY-DIF-3G and BODIPY-DIF-3R were synthesized as described in Materials and Methods. Molecular mass (Mr) of each compound is indicated in parentheses.

Fig. 2. Effects of BODIPY-DIF-3G and BODIPY-DIF-3R on HeLa cell growth, and cellular localization of the BODIPY-conjugated compounds. (A) Cells were incubated for 3 days with 0.2% dimethyl sulfoxide (DMSO; vehicle) or 20 μM of BODIPY-DIF-3G or BODIPY-DIF-3R, and relative cell number was assessed. Mean values and s.d. (bars) of three independent experiments are presented. **P < 0.01 versus DMSO control. *P < 0.01 versus DMSO control. (B) HeLa cells were incubated for 3 days with 0.2% DMSO or 20 μM of DIF-3, BODIPY-DIF-3G, or BODIPY-DIF-3R, and observed by using phase-contrast microscopy. (C,D) Cells were incubated for 0.5 h (C) or 3 days (D) with BODIPY-DIF-3G (20 μM) or BODIPY-DIF-3R (20 μM), washed free of the additives, and observed by using phase-contrast and fluorescence microscopy. Mitochondrial swelling (arrows) was induced in most of the cells treated with BODIPY-DIF-3G. Scale bars: 100 μm (B), 50 μm (C,D).
Access to both biologically functional and nonfunctional fluorescent DIF-3 derivatives could be powerful tools for studying structure–effect relationships and imaging the cellular localization and function of DIF-like molecules.

**Cellular localization of BODIPY-DIF-3G and BODIPY-DIF-3R in HeLa cells**

We next compared cellular localization of BODIPY-DIF-3G and BODIPY-DIF-3R in HeLa cells. Cells incubated for 0.5 h with 20 μM BODIPY-DIF-3G rapidly incorporated the DIF-3 derivative into intracellular organelles that we previously identified as mitochondria (Fig. 2C) (Kubohara et al., 2013). Incubation with BODIPY-DIF-3R under the same conditions resulted in a similar cellular distribution (Fig. 2C). However, while BODIPY-DIF-3G induced mitochondrial swelling (disturbance of the intracellular membrane) after 3 days (Fig. 2D, arrows), BODIPY-DIF-3R scarcely disturbed the intracellular membrane (Fig. 2D).

We then took a more detailed look at the intracellular localization of BODIPY-DIF-3R and BODIPY-DIF-3G under the same conditions in live HeLa cells by using high-magnification fluorescence microscopy and two fluorescent probes for mitochondria, MitoTrackerG and MitoTrackerR. BODIPY-DIF-3R was confirmed to localize mainly in the mitochondria, as shown by its co-localization with MitoTrackerG (Fig. 3A), with no remarkable changes in mitochondrial morphology or cell morphology throughout the 3-day incubation period (Fig. 3B).

When cells were incubated for 0.5 h with 20 μM each of BODIPY-DIF-3G and BODIPY-DIF-3R, the two compounds were confirmed to co-localize to the mitochondria (Fig. 4). Incubation for 3 days with 20 μM BODIPY-DIF-3G induced many mitochondria to swell, which were stained well with BODIPY-DIF-3G and MitoTrackerR (Fig. 5A) but were not stained with BODIPY-DIF-3R (Fig. 5B). In contrast, the morphologically normal, but not swollen, mitochondria were stained with both BODIPY-DIF-3G and BODIPY-DIF-3R (Fig. 5B).
Cellular localization of BODIPY-DIF-3G and BODIPY-DIF-3R in HeLa cells pre-treated with Bu-DIF-3 and carbonyl cyanide m-chlorophenyl hydrazine (CCCP)

Bu-DIF-3 is one of the more potent anti-tumor agents among the DIF-like molecules (Gokan et al., 2005), and it has been shown to induce mitochondrial swelling (Kubohara et al., 2013). Therefore, we compared the cellular localization of BODIPY-DIF-3G and BODIPY-DIF-3R in cells treated with 5 μM Bu-DIF-3. After 3 days, mitochondria swelled greatly in all the cells that were stained with BODIPY-DIF-3G and MitoTrackerR (Fig. 6A), but again, BODIPY-DIF-3R stained only the normal, but not swollen mitochondria (Fig. 6B).

CCCP is a mitochondrial uncoupler (proton-specific ionophore) that induces mitochondrial swelling in HeLa cells (Kubohara et al., 2013). We next compared the cellular localization of BODIPY-DIF-3G and BODIPY-DIF-3R in CCCP-treated cells. When cells were incubated for 3 days with 10 μM CCCP, mitochondria swelled greatly in all the cells that were stained with BODIPY-DIF-3G and MitoTrackerR (Fig. 6A), but again, BODIPY-DIF-3R stained only the normal mitochondria (Fig. 6B).

These results show that BODIPY-DIF-3R can penetrate the cell membrane and localize to normal mitochondria, but not to swollen mitochondria induced with the bioactive DIF-3 derivatives BODIPY-DIF-3G or Bu-DIF-3. Functionally, BODIPY-DIF-3R does not induce mitochondrial swelling. In addition, it is likely that the swollen mitochondria are morphologically, biochemically, and thus functionally different, from normal mitochondria and can be distinguished by staining with BODIPY-DIF-3G (or MitoTracker dyes) and BODIPY-DIF-3R.

Effects of DIFs on mitochondrial O2 consumption

We have previously shown that the bioactive compounds DIF-3, Bu-DIF-3, and BODIPY-DIF-3G act like mitochondrial uncouplers such as CCCP in that they also increase mitochondrial O2 consumption (Kubohara et al., 2013). We examined the effects of BODIPY-DIF-3R on mitochondrial O2 consumption in isolated mouse liver mitochondria in vitro by using a Clark-type oxygen electrode. As described previously (Kubohara et al., 2013), BODIPY-DIF-3G at 20–100 μM increased basal O2 consumption in a dose-dependent manner (Fig. 8). In contrast, the non-bioactive compound, BODIPY-DIF-3R, had a significant effect up to the maximum 100 μM dose. These results suggest that the inability of BODIPY-DIF-3R to suppress cell proliferation is due to its inability to disturb mitochondrial O2 consumption.

Discussion

In addition to D. discoideum being studied as a model organism in cell and developmental biology, other cellular slime molds have recently been shown to produce many pharmacologically active...
Fig. 6. Cellular localization of BODIPY-DIF-3G and BODIPY-DIF-3R in HeLa cells treated for 3 days with Bu-DIF-3 and carbonyl cyanide m-chlorophenyl hydrazine (CCCP). Cells were incubated for 3 days with Bu-DIF-3 (5 μM) (A,B) or CCCP (10 μM) (C,D), washed free of the additive, and further incubated for 0.5 h with BODIPY-DIF-3G (20 μM), Hoechst (0.1 μg/ml), and MitoTrackerR (0.1 μM) (A,C) or BODIPY-DIF-3R (20 μM) (B,D). Cells were washed free of the additives and observed by using high-magnification fluorescence microscopy. Three-dimensional (3D) images were constructed from z-stacked two-dimensional (2D) images, and two representative 2D-projections of the 3D images are shown. BODIPY-DIF-3G and MitoTrackerR co-localized to mitochondria, and the swollen mitochondria were stained with BODIPY-DIF-3G and MitoTrackerR, but not BODIPY-DIF-3R. Scale bars: 20 μm.

Fig. 7. Cellular localization of BODIPY-DIF-3G and BODIPY-DIF-3R in formalin-fixed HeLa cells. Cells were incubated for 3 days without (A) or with (B) CCCP (10 μM) and then incubated for a further 0.5 h with Hoechst (0.1 μg/ml) and MitoTrackerDR (0.2 μM). Cells were washed free of the additives and fixed with 3.7% formalin. The fixed cells were then stained for 0.5 h with BODIPY-DIF-3G (20 μM) and BODIPY-DIF-3R (20 μM), washed free of the additives, and observed by using high-magnification fluorescence microscopy. Merged images (c,f) were constructed with images of cells stained with BODIPY-DIF-3G, BODIPY-DIF-3R, or Hoechst (e) and those stained with BODIPY-DIF-3G, BODIPY-DIF-3R, or MitoTrackerDR (f) with the use of pseudo colors. Scale bars: 20 μm.
It is important to note that swollen mitochondria induced by BODIPY-DIF-3G, Bu-DIF-3, or CCCP could be stained with BODIPY-DIF-3G, but not with BODIPY-DIF-3R (Figs 5, 6), which was the case even in formalin-fixed cells (Fig. 7), suggesting that swollen mitochondria are functionally, biochemically, and/or biophysically different from normal mitochondria. Elucidation of these differences (e.g. by the use of the two BODIPY-DIF-3 derivatives described in this study) may reveal an unidentified target protein of functional DIF-like molecules in mitochondria.

**MATERIALS AND METHODS**

**Reagents and cell culture**

MitoTracker® Green FM (designated MitoTrackerG; Ex=490 nm, Em=516 nm), MitoTracker® Red CMXRs (MitoTrackerR; Ex=579, Em=599 nm), MitoTracker® Deep Red FM (MitoTrackerDR; Ex=644, Em=665 nm), BODIPY™ FL, SE (succinimidyl ester) (Ex=505 nm, Em=513 nm), and BODIPY™ TMR-X, SE (Ex=544 nm, Em=570 nm) were purchased from Invitrogen (Eugene, OR, USA). Hoechst 33342 (Ex=352 nm, Em=461 nm) solution (1 mg/ml H2O) and CCCP were obtained from Wako Pure Chemical Industries (Osaka, Japan). DIF-3 and Bu-DIF-3 were synthesized as described previously (Gokan et al., 2005) and stored as 10 mM solutions in DMSO at −20°C.

**Human cervical cancer HeLa cells** (a kind gift from Dr T. Oda, Gunma University, Japan) (Sekimoto et al., 2010) were maintained in vitro at 37°C (5% CO2 and 95% air) in DMEM-FBS [Dulbecco’s Modified Eagle’s Medium (DMEM) containing 4500 mg/l of glucose (Sigma, DS5796) supplemented with 75 μg/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum (FBS)].

**Synthesis of BODIPY-DIF-3G and BODIPY-DIF-3R**

BODIPY-DIF-3G was synthesized as described previously (Kubohara et al., 2013), and BODIPY-DIF-3R was synthesized in six reaction steps as described below (Fig. 1B). The synthesized compounds were stored as 10 mM solutions in DMSO at −20°C.

**Step 1. Synthesis of 5-(4-bromobutoxy)resorcinol**

Potassium carbonate (7.17 g, 51.9 mmol) and 1,4-dibromobutane (3.15 ml, 26.4 mmol) were added to a solution of chlorogluconic (4.20 g, 25.9 mmol) in N,N-dimethylformamide (100 ml) at room temperature. The reaction mixture was stirred for 2 h at 50°C, then diluted with 1.0 M hydrochloric acid (200 ml) and extracted with ethyl acetate (250 ml) three times. The combined organic layer was then washed with water (200 ml) and saturated sodium chloride solution (200 ml), dried over sodium sulfate, and evaporated under reduced pressure. The residue was chromatographed over a silica gel column with a hexane-ethyl acetate (2:1) solvent system to give 5-(4-bromobutoxy)resorcinol (1.94 g, 7.43 mmol).

**Step 2. Synthesis of 1-(4-(4-bromobutoxy)-2,6-dihydroxyphenyl)hexan-1-one**

Hexanoyl chloride (0.720 ml, 5.15 mmol) and aluminum chloride (1.34 g, 10.1 mmol) were added to a solution of 5-(4-bromobutoxy)resorcinol (1.31 g, 5.03 mmol) in dichloromethane (30 ml). The reaction mixture was stirred for 3 h at room temperature, then diluted with water (100 ml) and extracted with ethyl acetate (150 ml) three times. The combined organic layer was then washed with saturated sodium bicarbonate solution (150 ml) and saturated sodium chloride solution (150 ml), dried over sodium sulfate, and evaporated under reduced pressure. The residue was chromatographed over a silica gel column with a hexane-ethyl acetate (9:1) solvent system to give 1-(4-(4-bromobutoxy)-2,6-dihydroxyphenyl)hexan-1-one (0.910 mg, 2.53 mmol).

**Step 3. Synthesis of 1-(4-(4-bromobutoxy)-3-chloro-2,6-dihydroxyphenyl)hexan-1-one**

Sulfuryl chloride (342 mg, 2.53 mmol) was added to a solution of 1-(4-(4-bromobutoxy)-2,6-dihydroxyphenyl)hexan-1-one (910 mg, 2.53 mmol) in chloroform–ethanol (49:1) (25 ml). The reaction mixture was stirred for 1 h...
at room temperature and then evaporated under reduced pressure. The residue was chromatographed over a silica gel column with a hexane–ethyl acetate (9:1) solvent system to give 1-(4-(4-bromobutoxy)-3-chloro-2,6-dihydroxyphenyl)hexan-1-one (899 mg, 2.28 mmol).

Step 4. Synthesis of 1-(4-(4-azidobutoxy)-3-chloro-2,6-dihydroxyphenyl)hexan-1-one

Sodium azide (223 mg, 3.42 mmol) was added to a solution of 1-(4-(4-bromobutoxy)-3-chloro-2,6-dihydroxyphenyl)hexan-1-one (337 mg, 0.855 mmol) in N,N-dimethylformamide (8 ml) at room temperature. The reaction mixture was stirred for 3 h, and then diluted with water (30 ml) and extracted with ethyl acetate (40 ml) three times. The combined organic layer was washed with water (40 ml) and saturated sodium chloride solution (40 ml), dried over sodium sulfate, and evaporated under reduced pressure. The residue was chromatographed over a silica gel column with a hexane–ethyl acetate (4:1) solvent system to give 1-(4-(4-azidobutoxy)-3-chloro-2,6-dihydroxyphenyl)hexan-1-one (302 mg, 0.849 mmol).

Step 5. Synthesis of 1-(4-(4-aminobutoxy)-3-chloro-2,6-dihydroxyphenyl)hexan-1-one hydrochloride

Five percent palladium on carbon (2.0 mg) was added to a solution of 1-(4-(4-aminobutoxy)-3-chloro-2,6-dihydroxyphenyl)hexan-1-one (52 mg, 0.145 mmol) in 3% (w/v) hydrochloride methanol solution (3 ml) at room temperature. The reaction mixture was stirred for 1 h under a hydrogen atmosphere and then filtered through a Celite pad. The Celite pad was washed with methanol and the filtrate was evaporated under reduced pressure. The residue was chromatographed over a silica gel column with a chloroform–methanol (4:1) solvent system to give 1-(4-(4-aminobutoxy)-3-chloro-2,6-dihydroxyphenyl)hexan-1-one hydrochloride (52 mg, 0.142 mmol).


1-(4-(4-Aminobutoxy)-3-chloro-2,6-dihydroxyphenyl)hexan-1-one hydrochloride (7.9 mg, 21.5 µmol) and triethylamine (20 µl) were added to a solution of BODIPY-TMR-X, SE (2.6 mg, 4.3 µmol) in tetrahydrofuran (1 ml) at room temperature in the dark. The reaction mixture was stirred for 5 h, and then diluted with 0.2 M hydrochloric acid (5 ml) and extracted with ethyl acetate (10 ml) three times. The residue was subjected to recrystallization 

Preparation of mitochondria-enriched cell fraction and measurement of O2 consumption

Mitochondria were isolated from mouse liver (ICR, 7–10-week-old females) by differential centrifugation as described previously (Kabuyama et al., 2010; Kubohara et al., 2013). Mitochondrial O2 consumption was determined by using a Clark-type oxygen electrode (Strathkelvin Instruments Ltd., North Lanarkshire, Scotland) as described (Gottlieb et al., 2002; Wegryn et al., 2009). The mitochondria-enriched fraction was incubated in O2 measurement buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 0.1 mM EDTA, 5 mM phosphate, 5 mM glutamate, 20 mM Tris-HCl; pH 7.4) in the presence of 5 mM succinate, 5 mM glutamate, 20 mM Tris-HCl; pH 7.4) in the presence of 1% DMSO, BODIPY-DIF-3G (20 or 100 µM), or BODIPY-DIF-3R (20 or 100 µM) at 30°C. After recording the mitochondrial respiration State 4 reaction, an aliquot of ADP was added to a final concentration of 200 µM to induce State 3 respiration. However, since DIF-related molecules have been shown to affect State 4 respiration (Kubohara et al., 2013), the rate of O2 consumption during State 4 was calculated and compared in this study.

Statistics

Significance was assessed by unpaired (two-tailed) Student’s t-test. Values were considered significantly different when the P value was less than 0.05.

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Competing interests

The authors have no competing interests to declare.

Author contributions

Y.K., H.K., Y.O. and Y.H. conceived and designed the experiments; Y.K., H.K., Y.O. and Y.H. performed the experiments; Y.K. analyzed the data; and Y.K., H.K. and Y.H. wrote the paper.
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References


