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RESEARCH PAPER

Br-DIF-1 accelerates dimethyl sulphoxide-induced differentiation of P19CL6 embryonic carcinoma cells into cardiomyocytes

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BACKGROUND AND PURPOSE

Stem cell transplantation therapy is a promising option for treatment of severe ischaemic heart disease. Dimethyl sulphoxide (DMSO) differentiates P19CL6 embryonic carcinoma cells into cardiomyocyte-like cells, but with low differentiation capacity. To improve the degree of this differentiation, we have assessed several derivatives of the differentiation-inducing factor-1 (DIF-1), originally found in the cellular slime mould *Dictyostelium discoideum,* on P19CL6 cells.

EXPERIMENTAL APPROACH

P19CL6 cells were cultured with each derivative and 1% DMSO for up to 16 days. Differentiation was assessed by measuring the number of beating and non-beating aggregates, and the expression of genes relevant to cardiac tissue. The mechanism of action was investigated using a T-type Ca^{2+} channel blocker.

KEY RESULTS

Of all the DIF-1 derivatives tested only Br-DIF-1 showed any effects on cardiomyocyte differentiation. In the presence of 1% DMSO, Br-DIF-1 (0.3–3 μM) significantly and dose-dependently increased the number of spontaneously beating aggregates compared with 1% DMSO alone, by day 16. Expression of mRNA for T-type calcium channels was significantly increased by Br-DIF-1 + 1% DMSO compared with 1% DMSO alone. Mibefradil (a T-type Ca^{2+} channel blocker; 100 nM) and a small interfering RNA for the T-type Ca^{2+} channel both significantly decreased the beating rate of aggregates induced by Br-DIF-1 + 1% DMSO.

CONCLUSIONS AND IMPLICATIONS

Br-DIF-1 accelerated the differentiation, induced by 1% DMSO, of P19CL6 cells into spontaneously beating cardiomyocyte-like cells, partly by enhancing the expression of the T-type Ca^{2+} channel gene.

Abbreviations

ANP, atrial natriuretic peptide; BMP2, bone morphogenetic protein 2; Ca_v, voltage-dependent calcium channel; DIF, differentiation-inducing factor; FBS, fetal bovine serum; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; HCN, potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel; MHC; myosin heavy chain; MLC, myosin light chain; siRNA, small interfering RNA

Introduction

Reperfusion therapy for acute myocardial infarction is currently popular and known to reduce infarct size, improve left ventricular function and reduce mortality. However, these beneficial effects are limited by the acceleration of ischaemia-reperfusion injury, especially inflammation (Frangogiannis *et al.*, 2002; Moens *et al.*, 2005). Regenerative medicine aims to develop new medical treatments instead of reperfusion therapy for myocardial infarction and heart failure. The enhancement of stem cell differentiation into cardiomyocytes by a bioactive drug may become a useful strategy, conditional on its success in clinical trials of myocardial infarction and heart failure (Soignet *et al.* 2000). Furthermore, these drugs provide versatile experimental probes to understand mechanisms that determine cell fate. Exploration of new efficient inducers of cardiomyocyte differentiation is of great importance (Ohtsu *et al.*, 2005; Sadek *et al.*, 2008).

The P19CL6 embryonal carcinoma cell line is derived from a teratocarcinoma formed in C3H/He mice (Martin, 1975), and has been used successfully as a model system for cardiomyocyte differentiation (McBurney *et al.*, 1982). A significant experimental advantage of P19CL6 cells compared to embryonic stem cells is that they can sustain an undifferentiated cell state without a feeder-cell layer, making them much easier to culture. Therefore, this cell line has been used extensively to assay the induction of cardiomyocyte differentiation by various molecules (Martin, 1975; McBurney *et al.*, 1982; Habara-Ohkubo, 1996; Choi *et al.*, 2004; Gassanov *et al.*, 2008). Dimethyl sulphoxide (DMSO) has primarily been used to induce cardiomyocyte differentiation in P19CL6 cells (McBurney *et al.*, 1982; Habara-Ohkubo, 1996). Several effective drugs such as retinoic acid and 5-azacytidine were developed using P19CL6 cells (Choi *et al.*, 2004; Gassanov *et al.*, 2008). However, the low potency of these inducers reduces their usefulness. The development of novel compounds that provide a much higher degree of differentiation is ongoing.

The differentiation-inducing factor-1 (DIF-1) is relatively simple chemical compound (1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone]; Figure 1) (Morris *et al.*, 1987) and is a putative morphogen that induces stalk cell formation in the cellular slime mould, *Dictyostelium discoideum* (Kay *et al.*, 1989). Recently, it was found that DIF-1 and its derivatives suppress cell growth and induce erythroid differentiation in murine and human leukaemia (B8 and K562) cells (Asahi *et al.*, 1995; Kubohara, 1999; Gokan *et al.*, 2005). In particular, co-administration of retinoic acid and DIF-1 or its synthetic derivatives may improve chemoprevention/differentiation therapy for acute promyelocytic (and other types of) leukaemia (Kubohara, 1997). Given the differentiation effects of DIF-1 in leukaemia cells, it is possible that similar effects could occur in other multipotent stem cells such as carcinoma and mesenchymal stem cells. This study was designed to determine whether DIF-1 and its derivatives induced cardiomyocyte differentiation in the P19CL6 carcinoma cell line. We show here that DIF-1 did not induce the differentiation of P19CL6 cells; however, Br-DIF-1 (in which two chlorine atoms in DIF-1 are replaced with bromine) accelerated the 1% DMSO-induced differentiation of P19CL6 into spontaneously beating cardiomyocyte-like cells by enhancing the expression of the gene for the T-type calcium channel, $Ca_v3.1$.

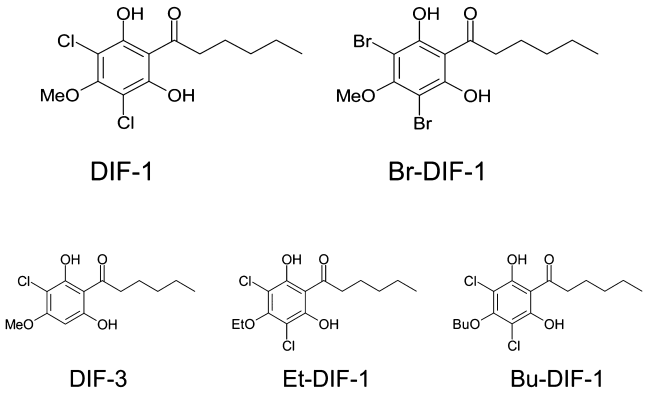

Figure 1

Structures of DIF-1 and its derivatives. Br-DIF-1, where the two chlorines in DIF-1 were replaced with bromine. DIF-3, where one chlorine in DIF-1was replaced with a hydroxyl. Et-DIF-1, where the methoxy group in DIF-1 was replaced with an ethoxy group. Bu-DIF-1, where the methoxy group in DIF-1 was replaced with a n-butoxy group.

Methods

Culture and cardiomyocyte differentiation of P19 cells

P19CL6 cells were obtained from the RIKEN BRC cell bank (Tsukuba, Japan) and cultured in α-MEM supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. All cells were passaged at 48 h intervals. To induce differentiation, the cells were cultured at a density of 2.0×10^4 cells per 35 mm dish in medium containing 1% DMSO as a positive control, DIF-1 (1–3 μM) and Br-DIF-1 (0.3–3 μM). The medium containing reagents was replenished at 48 h intervals. After treatments with reagents, the morphological changes in P19CL6 cells were examined under a Nikon inverted microscope (Nikon, Tokyo, Japan) equipped with a phase-contrast objective, a digital camera (Nikon, Tokyo, Japan) and a video camera (Canon, Tokyo, Japan). For immunocytochemical staining, after formaldehyde fixation, cells were analysed under a fluorescence microscope (IX71; Olympus, Tokyo, Japan) and a confocal microscope system (ECLIPSE C1si; Nikon Instruments Inc., Tokyo, Japan).

Aggregates formed after 8 days of culture and subsequently increased time-dependently (Figure S1). These aggregates were positive for α-myosin heavy chain (α-MHC) antibody, as determined by immunofluorescence microscopy (Figure 2). We measured numbers of aggregates (as an index of differentiation into cardiomyocyte-like cells) and beating aggregates (as an index of differentiation into spontaneously beating cardiomyocyte-like cells), scoring every 2 days using a phase-contrast microscope (Deb *et al.*, 2008). Images (×100) from five separate visual fields were obtained from each insert and the number of aggregates was evaluated. These measure-

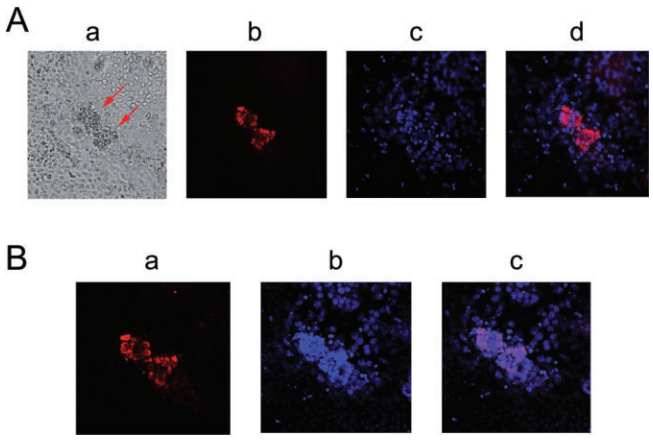

Figure 2

Typical fluorescent images of aggregates formed by P16CL6 cells. Representative images of anti-α-myosin heavy chain (MHC) monoclonal antibody/Alexa 568 anti-mouse IgG and Hoechst 33258 staining of aggregates formed by P19CL6 cells in the presence of 1% DMSO and Br-DIF-1 (3 μM) over 16 days. (A) Images using a fluorescence microscope, IX71. a: phase-contrast image of two aggregates after formaldehyde fixation (arrows), b: fluorescent image of α-myosin heavy chain- and Alexa 568-positive aggregates, c: fluorescent image of cell nuclei stained with Hoechst 33258; d: merged image of b and c. (B) Fluorescent images using a confocal fluorescence microscope, ECLIPSE C1si. a: α-myosin heavy chain- and Alexa 568-positive aggregates, b: cell nuclei stained with Hoechst 33258, and c: merged image of a and b.

ments were made only for 16 days because, in our experimental conditions, extending incubation beyond this time resulted in degeneration of aggregates and loss of aggregate rhythmicity.

Measurement of gene expression

Total RNA was isolated from cells using a QuickGene RNA cultured cell kit S (Fuji Film, Tokyo, Japan). An aliquot of total RNA was reverse transcribed using random primers. For real-time PCR reactions, cDNA was amplified (ABI PRISM 7000, Life Technologies, Carlsbad, CA, USA) under the following reaction conditions: 40 cycles of PCR (95°C for 15 s, and 60°C for 1 min) after an initial denaturation (95°C for 10 min). The reaction volume was adjusted to 26 μL containing 3 μL of a 1:4 dilution of the first-strand reaction product, 1 μL of 10 μM specific forward and reverse primers, 8 μL of pure water, and 13 μL of SYBR Green. The primers used for real-time PCR were designed by Primer Express (Applied Biosystems) and their sequences are listed in Table 1. Amplification of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), served as a normalization standard. Gene expression was quantified as the fold change in expression of each gene relative to expression on Day 0.

The effect of mibefradil on Br-DIF-1-induced differentiation of P19CL6 into cardiomyocyte-like cells

P19CL6 cells were cultured in the presence of 1% DMSO with or without Br-DIF-1 (3 μM) for 16 days prior to addition of mibefradil (100 nM), a T-type calcium channel blocker, and a further 3 h incubation. To assess the effects of mibefradil, changes in the numbers of beating aggregates and the beating rate of each cardiomyocyte-like aggregate were measured using a phase-contrast microscope. Data for each experiment are the average values of 10–20 aggregates.

The effect of $Ca_v3.1$ siRNA on Br-DIF-1-induced differentiation of P19CL6 into cardiomyocyte-like cells

P19CL6 cells were cultured at a density of 4000 cells per 15 mm dish for 5 h. Cells were treated with control siRNA and $Ca_v3.1$ siRNA (100 pM) using MultiFectam transfection reagent (Promega Co., WI, USA) according to the manufacturer's instructions. After 4 h of transfection, cells were cultured in the presence of 1% DMSO and Br-DIF-1. Transfection media was replenished at 4 day intervals. Real-time PCR was used to verify that siRNA decreased T-type Ca^{2+} channel gene expression.

Statistical analysis

All statistical analyses were carried out by KyPlot 5.0 software (Kyenslab, Tokyo, Japan). Group comparisons were performed by ANOVA with the Student–Newman–Keuls *post hoc* correction procedure or with Student's *t*-test. Values are presented as mean ± SEM; $P < 0.05$ was considered to indicate statistical significance.

Materials

Mibefradil dihydrochroride was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). α-Minimal essential medium (α-MEM), penicillin and streptomycin were purchased from Invitrogen (Grand Island, NY, USA). Control small interfering RNA (siRNA) and Cav3.1 siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Fetal bovine serum (FBS) was obtained from Nichirei Biosciences (Tokyo, Japan). Other analytical grade chemicals were obtained from Wako Pure Chemicals (Osaka, Japan). Stock solutions of chemicals were freshly made prior to each experiment, and at high concentrations to minimize any vehicle effects. Synthesis of DIF-1, Br-DIF-1 and their derivatives were performed using methods described previously (Gokan *et al.*, 2005). The structures of these compounds were determined by IR and UV spectroscopy, fast-atom bombardment mass spectrometry and ^{1}H- and ^{13}C-NMR spectra (see Figure 1). The DIF-1 and Br-DIF-1 were completely pure as assessed by ^{1}H- and ^{13}C-NMR spectra. All synthetic products were freshly dissolved in DMSO. The final concentration of DMSO in the experimental tubes never exceeded 0.1% and had no effect on the cells or assays.

Results

Effect of Br-DIF-1 on 1% DMSO-induced differentiation of P19CL6 cells into spontaneously beating cardiomyocyte-like cells

When P19CL6 cells differentiate into spontaneously beating cardiomyocyte-like cells, beating aggregates are produced

Table 1

Primers used for quantitative real-time PCR

Gene symbol	Genbank accession No.	Sequences (5′–3′)
G3PDH	AF106860	Forward:TGCACCACCAACTGCTTAG
		Reverse: GGATGCAGGGATGATGTTC
α-MHC	NM_010856	Forward: CATGGCTACACTCTTCTCTACCTATGC
		Reverse: GAGCAGACACTGTTTGGAAGGA
GATA4	NM_008092	Forward: CCAATTTGGGATTTTCTTTTTCC
		Reverse: CCAACTCGCTCAAAATATATACGAATTA
BMP2	NM_007553	Forward: ACACAGGGACACACCAACCAT
		Reverse: TGTGACCAGCTGTGTTCATCTTG
Cav1.2	AB259049	Forward: GAGCCACGGTGAATCAGGA
		Reverse: GCAGTACTCGGCTTCTTCACTCA
Cav1.3	AJ437292	Forward:TACGTGGTGAACTCCTCGCCTTTCGAAT
		Reverse: CCAGGCAGAGCGTGTTGA
Cav3.1	AJ012569	Forward: CCTGAGAATTTCAGCCTCCC
		Reverse: GATCGCATGCCGTTCTCC
Cav3.2	NM_021415	Forward: ATGTACTCACTGGCTGTGACC
		Reverse: GAGTCCAAAAGAGTGTGGGC
HCN2	NM_008226	Forward: CGAGGTGCTGGAGGAATACC
		Reverse: TGCGATCTAGCCGGTCAATAG
HCN4	AF064874	Forward: CGGCAAGAAGATGTACTTTATCCA
		Reverse: TTGGTCTCTTTGTTGCCCTTAGT
MLC2a	NM_022879	Forward: CAGGAATTCAAGGAAGCCTTCA
		Reverse: AGCTGGGAATAGGTCTCCTTCAG
MLC2v	NM_010861	Forward: GGACGAGTGAACGTGAAAAATG
		Reverse: GAGGAACACGGTGAAGTTAATTGG
ANP	NM_008725	Forward: TTCCTCGTCTTGGCCTTTTG
		Reverse: GGTCTAGCAGGTTCTTGAAATCCA

(Supporting Information Video S1). DMSO (1%; positive control) induced the formation of spontaneously beating aggregates on Day 8 and the average number of spontaneously beating aggregates was 25.5 ± 5.2 on Day 16, compared to zero in control cells (Figure 3A). DIF-1 or Br-DIF-1 alone, at concentrations up to 3μM, did not produce spontaneously beating aggregates up to Day 16. In contrast, the number of spontaneously beating aggregates induced by 1% DMSO on Day 16 was significantly and dose-dependently facilitated in the presence of Br-DIF-1 (3 μM; $P < 0.01$ vs. 1% DMSO; Figure 3B), but not by DIF-1 (3 μM, 27.0 ± 2.0) At lower concentrations of Br-DIF-1, the number of spontaneously beating aggregates induced by 1% DMSO was 29.3 ± 1.3 for 0.3 μM Br-DIF-1 and 40.8 ± 5.2 for 1 μM Br-DIF-1, on Day 16. Lower concentrations of DMSO (0.1 and 0.3%) with or without Br-DIF-1 failed to induce spontaneously beating aggregates.

Gene expression of pacemaker channels

Spontaneously beating pacemaker cells like sinoatrial nodal cells express certain characteristic genes such as those for L-type ($Ca_v1.2$ and $Ca_v1.3$) and T-type ($Ca_v3.1$ and $Ca_v3.2$) calcium channels and the hyperpolarization-activated cation channels (HCN2 and HCN4). We investigated the expression of these genes to confirm the mechanism by which Br-DIF-1 caused acceleration of the production of spontaneously beating aggregates induced by 1% DMSO.

On Day 14, expression of $Ca_v3.1$ (Figure 4A) and HCN4 (Figure 4B) was significantly decreased in the presence of 1% DMSO, compared with control. The combination of 1% DMSO and Br-DIF-1 (3 μM) significantly reversed the 1% DMSO-induced deficit in $Ca_v3.1$ expression ($P < 0.05$ vs. 1% DMSO) but not that of HCN4 on Day 14. The expression of $Ca_v3.1$ induced by the non-effective compound, DIF-1 (3 μM), in the absence and presence of 1% DMSO, was 0.45 ± 0.11 and 0.38 ± 0.15 respectively, not different from the expression under control conditions (0.43 ± 0.05).

In contrast, 1% DMSO alone did not affect the expression of $Ca_v1.2$, $Ca_v1.3$, $Ca_v3.2$ and HCN2 genes (Figure 4B, 4C) on Day 14, compared with control. Br-DIF-1 (3 μM) did not alter this situation and $Ca_v1.2$, $Ca_v1.3$, $Ca_v3.2$ and

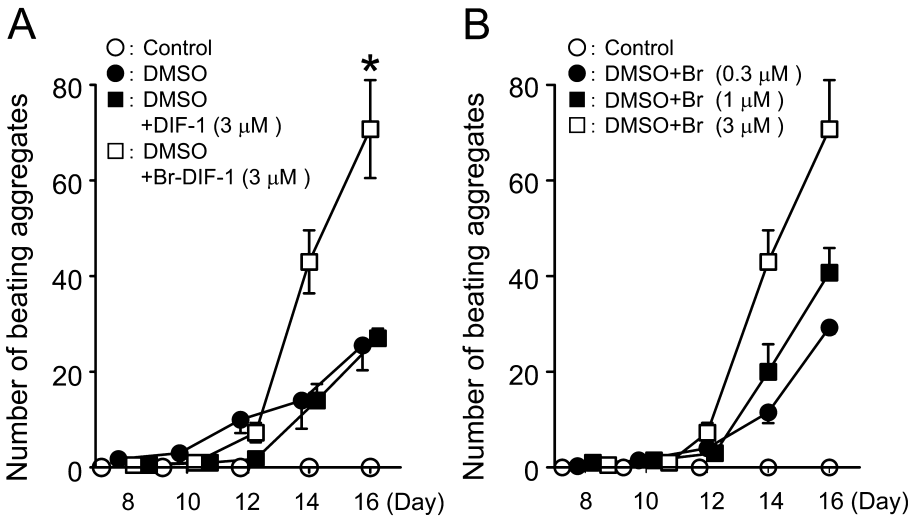

Figure 3

Br-DIF-1 but not DIF-1 increases the number of spontaneously beating aggregates differentiated from P19CL6 cells in the presence of 1% DMSO. (A) Time-course of changes in the number of spontaneously beating aggregates, an index of differentiation from P19CL6 cells into spontaneously beating cardiomyocyte-like cells. (B) Dose-dependent increase in the number of spontaneously beating aggregates by Br-DIF-1 (Br; 0.3, 1, 3 μM) in the presence of 1% DMSO; results from cultures with Br-DIF-1 alone are shown as Control. Values are mean ± SEM, $n = 4$. *$P < 0.05$ significantly different from 1% DMSO.

HCN2 genes in the presence of 1% DMSO and Br-DIF-1 (3 μM) was not significantly different to control.

Effect of Br-DIF-1 on induction of cardiomyocyte differentiation from P19CL6 cells by 1% DMSO

DMSO (1%) induced the formation of aggregates on Day 8 and reached a plateau on Day 14 (Figure 5A), about six times the control value (12.3 ± 4.3). Neither DIF-1 nor Br-DIF-1 alone (both at 3μM) induced cardiomyocyte differentiation of P19CL6 cells, up to Day 14. Aggregate formation induced by 1% DMSO on Day 16 was not significantly facilitated in the presence of either DIF-1 (3 μM) or Br-DIF-1 (3 μM). We also investigated the effects of other DIF-1 derivatives such as DIF-3, ethyl DIF-1 and butyl DIF-1 (Figure 1). However, these compounds failed to facilitate 1% DMSO-induced formation of either non-beating (Table 2) or spontaneously beating (data not shown) aggregates.

The expression of the cardiac-specific genes α-myosin heavy chain (MHC), GATA4 and bone morphogenetic protein 2 (BMP2) induced by 1% DMSO on Day 14 was very high (Figure 5B). relative to the values in the absence of 1% DMSO. Both DIF-1 and Br-DIF-1 (3 μM) alone significantly increased the expression of α-MHC ($P < 0.01$ vs. control) but not of GATA4 or of BMP2 on Day 14. However, this potentiation of α-MHC gene expression did not contribute to the cardiomyocyte differentiation from P19CL6 cells. The expression of cardiac-specific genes induced by 1% DMSO was not affected by the presence of DIF-1 and Br-DIF-1 (Figure 5B).

We further measured expression of other cardiac specific genes, myosin light chain (MLC)2a, MLC2v and atrial natriuretic peptide (ANP) induced by 1% DMSO in the presence or absence of Br-DIF-1 (3 μM) (Figure S2). DMSO (1%) induced significant expression of MLC2a ($P < 0.001$ vs. non-treatment) and MLC2v ($P < 0.001$ vs. non-treatment) on Day 14. However, these increases in gene expression were not altered by the presence of Br-DIF-1. On the other hand, P19CL6 cells spontaneously and significantly induced ANP gene expression on Day 14, and this expression was not accelerated by 1% DMSO, in the presence or absence of Br-DIF-1.

Effect of mibefradil on the Br-DIF-1-induced cardiac automaticity

The contribution of T-type calcium channels to the automaticity of the aggregates were pharmacologically investigated using mibefradil, a T-type calcium channel blocker, comparing the effects of mibefradil on the beating actions of P19CL6 cells induced by 1% DMSO in the presence and absence of Br-DIF-1. The initial beating rate of P19CL6 cells in the presence of 1% DMSO + Br-DIF-1 (3 μM) was significantly increased to 62.9 ± 5.1 beats per minute (bpm, $n = 38$, $P < 0.05$) on Day 16 compared to 1% DMSO alone (49.2 ± 3.8 bpm, $n = 38$). The time-dependent changes in beating rate and number of spontaneous beating aggregates in the presence of mibefradil were quantified relative to those values just before mibefradil administration (0 h), set to 100%. Although mibefradil (100 nM) did not decrease the number of beating aggregates (Figure 6A), this drug caused a significant and time-dependent decrease in the beating rate of each aggregate induced by 1%DMSO + Br-DIF-1 (3 μM), compared to the aggregates induced by 1%DMSO alone (Figure 6B, Videos S2,S3). The effect of mibefradil on the beating rate of the aggregates induced by 1% DMSO and Br-DIF-1 was also concentration-dependent. Thus, at lower concentrations (10 and 30 nM) of mibefradil, the beating rate of differentiated

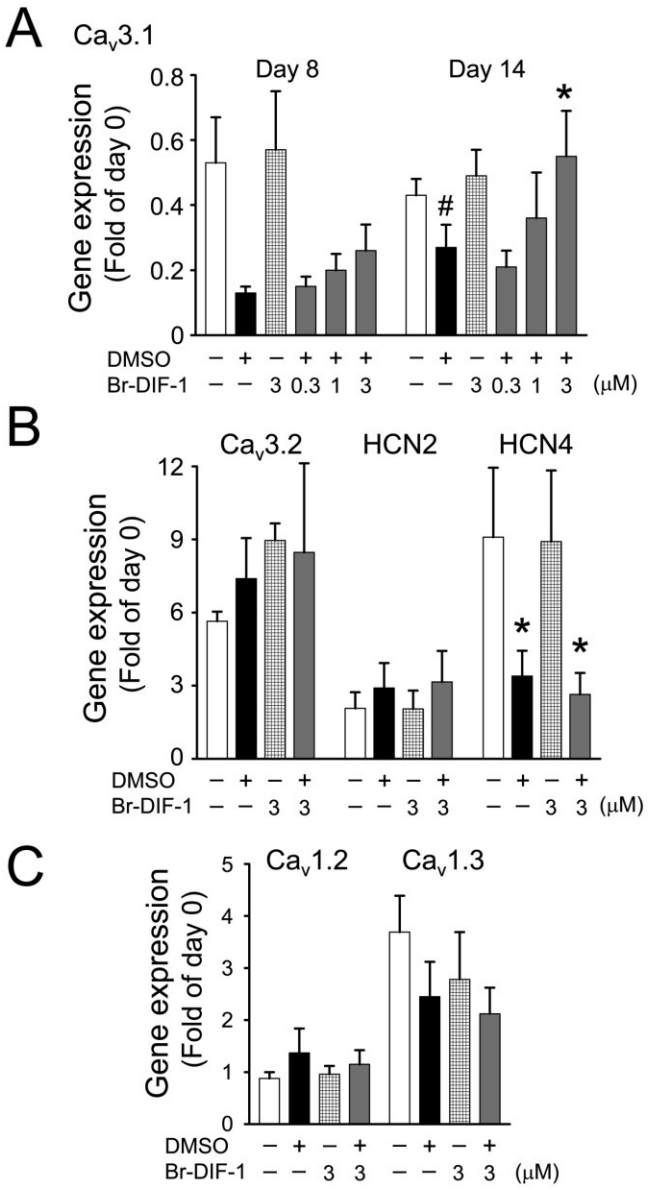

Figure 4

Br-DIF-1 regulates DMSO-induced changes in $Ca_v3.1$ gene expression. (A) Time-course of expression of the pacemaker channel gene, $Ca_v3.1$. Gene expression levels were normalized to the G3PDH gene. All ratios were calculated on Day 0. Bars represent the mean ± SEM, $n = 4$. *$P < 0.05$ significantly different from DMSO alone, $^{\#}P < 0.05$ significantly different from control on Day 14. (B) Expression of the pacemaker channel genes, $Ca_v3.2$, HCN2 and HCN4 on Day 14. Gene expression levels were normalized to the G3PDH gene. All ratios were calculated on Day 0. Open bars: control; closed bars: 1% DMSO; hatched bars: Br-DIF-1 (3 μM); gray bars: 1% DMSO + Br-DIF-1 (3 μM). Bars represent the mean ± SEM, $n = 4$. *$P < 0.05$ significantly different from control. (C) Expression of L-type calcium channel genes, $Ca_v1.2$ and $Ca_v1.3$ on Day 14. Gene expression levels were normalized to the G3PDH gene. All ratios were calculated on Day 0. Bars represent the mean ± SEM, $n = 4$.

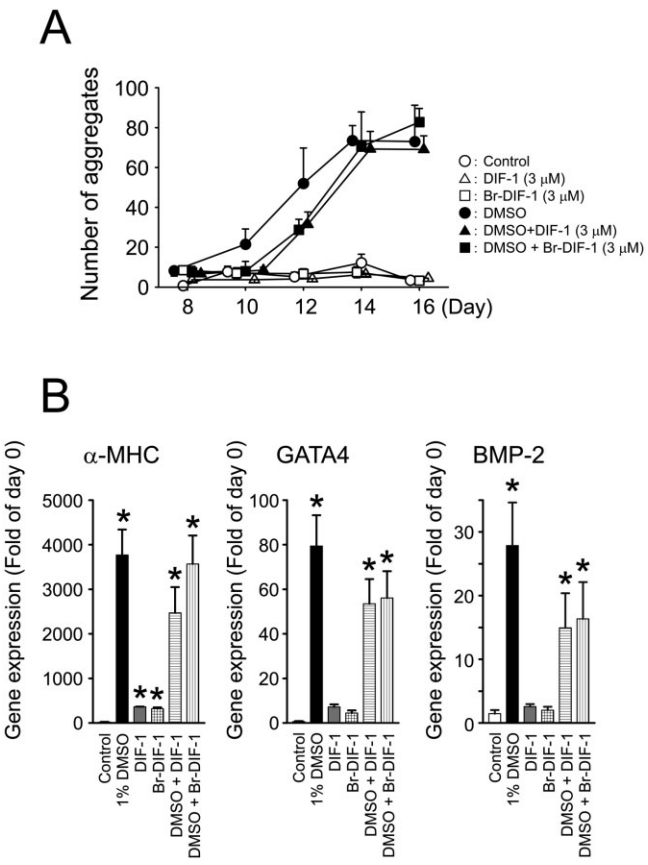

Figure 5

Neither DIF-1 nor Br-DIF-1 potentiates 1% DMSO-induced differentiation of P19CL6 cells into cardiomyocyte cells. (A) Time-course of changes in the number of aggregates, an index of cardiomyocyte differentiation from P19CL6 cells.. (B) Expression of cardiac specific genes α-MHC, GATA4 and BMP2 in P19CL6 cells on Day 14. Gene expression levels were normalized to the G3PDH gene. All ratios were calculated on Day 0. Bars represent the mean ± SEM, $n = 4$. *$P < 0.01$ significantly different from control.

cells was 68.4 ± 15.4% (n = 4) and 54.8 ± 13.6 % (n = 6), respectively, at 3 h.

Effect of $Ca_v3.1$ siRNA on the Br-DIF-1-induced cardiac automaticity

We further investigated the contribution of T-type calcium channels to the automaticity of the aggregates induced by 1% DMSO and Br-DIF-1 (3 μM) by using an siRNA which silences the expression of the T-type calcium channel, $Ca_v3.1$. Expression of the $Ca_v3.1$ gene was significantly decreased by the presence of $Ca_v3.1$ siRNA (100 pM), compared with that in cells without siRNA (P<0.05; Figure 7A). $Ca_v3.1$ gene expression after addition of scrambled siRNA (100 pM), as a negative control, was not changed by 1% DMSO + Br-DIF-1.

The number of aggregates induced by 1% DMSO + Br-DIF-1 (3 μM) on Day 14 was significantly decreased (P < 0.05) in the presence of $Ca_v3.1$ siRNA (100 pM) (Figure 7B). The number of beating aggregates induced by 1% DMSO + Br-DIF-1 (3 μM) was markedly decreased by $Ca_v3.1$ siRNA

Table 2

The number of aggregates differentiated from P19CL6 cells in the presence of 1% DMSO or various DIF-1 derivatives

Name	Number of aggregates on day 14 (DIF-1 derivative alone)	Number of aggregates on day 14 (in the presence of 1% DMSO)
NONE	12.3 ± 4.3	53.5 ± 7.5
DIF-1 (3 μM)	6.5 ± 1.2	69.3 ± 8.9
DIF-3 (3 μM)	3.0 ± 1.0	55.7 ± 9.7
Et-DIF-1 (3 μM)	1.6 ± 1.4	58.0 ± 5.4
Bu-DIF-1 (3 μM)	5.8 ± 3.2	61.6 ± 10.8
Br-DIF-1 (3 μM)	7.5 ± 2.8	75.5 ± 14.6

Values in the Table are means ± SEM from 4 cultures.

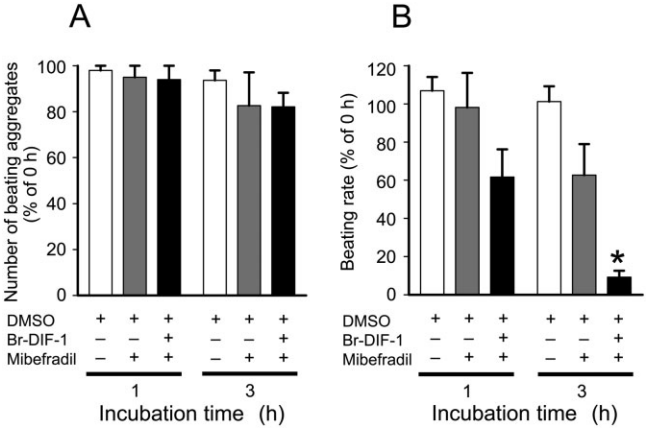

Figure 6

Mibefradil decreases the beating rate of each spontaneously beating cardiomyocyte-like cell differentiated by 1% DMSO + Br-DIF-1 (3 μM). (A) Time-course of changes in the number of beating aggregates produced by 1% DMSO + mibefradil (100 nM) and 1% DMSO + Br-DIF-1 with or without mibefradil (100 nM). (B) Time-course of changes in the beating rate of spontaneously beating aggregates produced by 1% DMSO + mibefradil (100 nM) and 1% DMSO + Br-DIF-1 with or without mibefradil (100 nM). Values are mean ± SEM, $n = 6$. *$P < 0.01$ significantly different from 1% DMSO + mibefradil at 3 h.

($P < 0.01$) but not by scrambled siRNA (Figure 7C). Furthermore, the beating rate of aggregates in the absence of siRNA was also significantly decreased by $Ca_v3.1$ siRNA ($P < 0.01$) but not by scrambled siRNA (Figure 7D).

Discussion and conclusions

We have demonstrated here that Br-DIF-1, a novel derivative of DIF-1 in which the chlorines are substituted by bromines, dose-dependently potentiated the effects of 1% DMSO on the

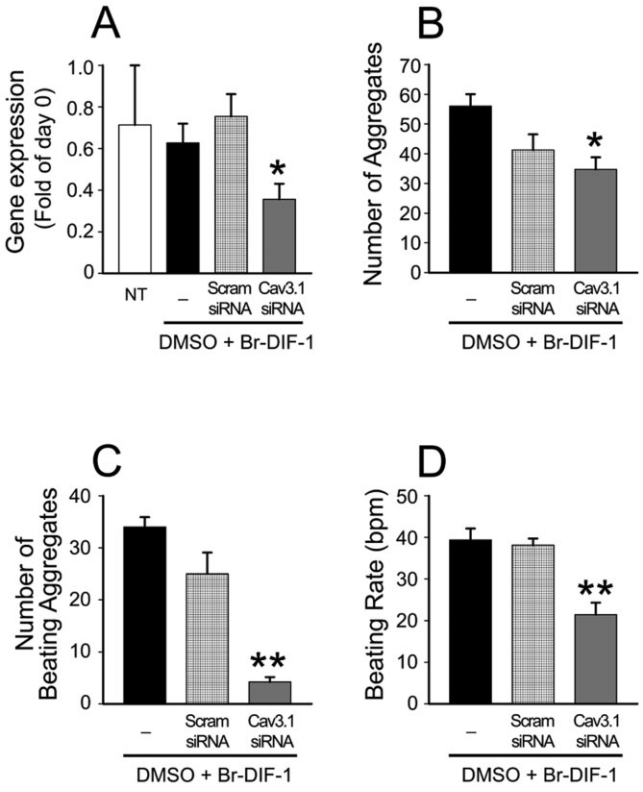

Figure 7

siRNA for $Ca_v3.1$ (100 pM) inhibits the beating rate of each spontaneously beating aggregate differentiated by 1% DMSO + Br-DIF-1 (3 μM). (A) Expression of $Ca_v3.1$ gene in P19CL6 cells on Day 14. Gene expression levels were normalized to G3PDH gene. All ratios were calculated on Day 0. No treatment (NT); scrambled siRNA (Scram siRNA) Bars represent the mean ± SEM, $n = 4$. *$P < 0.05$ significantly different from 1% DMSO + Br-DIF-1 in the presence or absence of scrambled siRNA. (B–D) Functional effects of $Ca_v3.1$ siRNA on the number of aggregates (B); number of spontaneously beating aggregates (C); and beating rate of spontaneously beating aggregates (D), induced by 1% DMSO + Br-DIF-1 (3 μM). Bars represent the mean ± SEM, $n = 4$. *$P < 0.05$ significantly different from 1% DMSO + Br-DIF-1, **$P < 0.01$ significantly different from 1% DMSO + Br-DIF-1 with or without scrambled siRNA.

differentiation of P19CL6 cells into beating cardiomyocyte-like cells. Br-DIF-1 reversed the decrease in $Ca_v3.1$ gene expression induced by by 1% DMSO alone. Mibefradil, a selective T-type calcium channel blocker, time-dependently decreased the beating rate of each spontaneously beating aggregate, while maintaining the overall number of beating aggregates. siRNA for the T-type calcium channel, $Ca_v3.1$, also significantly decreased the beating rate of aggregates induced by Br-DIF-1 and DMSO. These results suggest that Br-DIF-1 is effective at accelerating the differentiation of P19CL6 cells into spontaneously beating cardiomyocyte-like cells.

It is known that DIF-1 has anti-proliferative and differentiation-inducing effects on human cells. For example, DIF-1 inhibits the proliferation (namely, de-differentiation) of HL-60 human myeloid leukaemia cells (Kubohara, 1997) and human vascular smooth muscle cells by inhibiting the cell cycle (Miwa *et al.*, 2000). However, in

this study, DIF-1 alone did not induce the differentiation of P19CL6 cells into cardiomyocyte-like cells. Other DIF-1 derivatives containing chloride groups also failed to induce cardiomyocyte differentiation of P19CL6 cells (Kikuchi *et al.*, 2008). In contrast, Br-DIF-1 in combination with 1% DMSO unexpectedly potentiated differentiation of P19CL6 cells into spontaneously beating cardiomyocyte-like cells. Our results show that Br-DIF-1 reversed the DMSO-induced deficit in the expression of Cav3.1, a T-type Ca^{2+} channel gene. No other DIF-1 derivatives tested exerted this action. P19CL6 cells grown in the absence or presence of DIF-1 derivatives maintain low expression of $Ca_v3.1$, so insufficient differentiation into cardiomyocyte-like cells may prohibit their further differentiation into spontaneously beating cardiomyocyte-like cells. While this may explain the lack of effect of DIF-1 derivatives on differentiation into spontaneously beating aggregates, it does not explain why Br-DIF-1, but not DIF-1 or other DIF-1 derivatives such as DIF-3, ethyl DIF-1 and butyl DIF-1, was able to rescue the cells from the negative effects of 1% DMSO. The structural difference between Br-DIF-1 and other DIF-1 derivatives consists only of the replacement of a chloride group by a bromide group. Dehalogenation reactions are known to take place as an intracellular biotransformation in the microsomal fraction and the bromide ion produced will interact with Schiff base regions of various proteins, such as transcription factors (Noyes *et al.*, 2010). Further study of the role of bromide ions in the inhibition of $Ca_v3.1$ gene expression by DMSO is needed to clarify the particular differentiation mechanism of P19CL6 cells that is potentiated by Br-DIF-1.

Two ion channels have been shown to contribute to the pacemaker function of cardiac sinoatrial node cells: the T-type calcium channel ($Ca_v3.1$) with its associated current, $I_{Ca,T}$ and the hyperpolarization-activated cation channel (HCN) and its current I_f (Ohtsu *et al.*, 2005; Gassanov *et al.*, 2008). In this study, we confirmed that Br-DIF-1 regulates the expression of the $Ca_v3.1$ T-type calcium channel gene, but not the HCN gene. Br-DIF-1 did not affect expression of the two L-type calcium channels related to the contractile responses of cardiomyocyte-like cells. These results support previous data showing that the acceleration of $I_{Ca,T}$ channel activity contributes to spontaneous beating of cardiomyocyte-like cells induced by retinoic acid (Gassanov *et al.*, 2008).

To clarify the relationship between Br-DIF-1 and T-type calcium channels, we used mibefradil (a selective T-type calcium channel blocker) and $Ca_v3.1$ siRNA (siRNA of the T-type calcium channel). Mibefradil reduced the beating rate of each spontaneously beating aggregate in a dose-dependent manner, but not the overall number of beating aggregates. Although it is known that a high concentration (10 μM) of mibefradil inhibits not only T-type but also L-type calcium channel activity, the inhibition of L-type channels is greatly diminished at the concentration used here (100 nM) (Mishra and Hermsmeyer, 1994). Nifedipine (10–100 nM), a typical inhibitor of L-type calcium channels, dose-dependently inhibited the number of beating aggregates without any decrease in beating rate (data not shown). Although $Ca_v3.1$ siRNA also decreased the beating rate of aggregates induced by Br-DIF-1 and DMSO, its effect occurred together with the decrease in the number of beating aggregates. The negative chronotropic effect of $Ca_v3.1$ siRNA and mibefradil is the result of dysfunction of the T-type Ca^{2+} current (Mangoni *et al.*, 2006). Some investigators have reported that dysfunction of $Ca_v3.1$ channels during cardiac development affected the development of the cardiac conduction system (Mizuta *et al.*, 2010). Thus, the decrease in the number of beating aggregates by $Ca_v3.1$ siRNA may be caused by the dysfunction of conduction system generation in each aggregate. Our results therefore suggest that T-type calcium channels have a key role in the spontaneous beating of cardiomyocyte-like cells differentiated from P19CL6. In future work, we aim to demonstrate in more detail the mechanism by which Br-DIF-1 reverses the DMSO-induced decrease in $Ca_v3.1$ gene expression.

DMSO has been used to induce differentiation in a variety of human mesenchymal stem cells (Okura *et al.*, 2011; Hegner *et al.*, 2005). Our present study has demonstrated the possibility that Br-DIF-1 has a potentiating effect on the differentiation of P19CL6 cells into spontaneously beating cardiomyocyte-like cells induced by DMSO. Therefore, Br-DIF-1 should be able to supplement the effect of DMSO on cardiomyocyte differentiation of human mesenchymal stem cells. However, 1% DMSO (equivalent to ~128 mM) is a high concentration and has the potential problem of reducing clinical benefit because of its cytotoxicity (Ruiz-Delgado *et al.*, 2009). While Br-DIF-1 may be used to reduce the amount of DMSO required for differentiation, it is likely that, for the clinical application of Br-DIF-1, further investigation in other stem cell lines and with other differentiation inducers will be necessary. Indeed, we have already begun investigating the differentiation-inducing effects of Br-DIF-1 on the cardiac differentiation of other human multipotent cell lines, and will also determine whether its potentiating effects in cardiomyocyte differentiation apply to differentiation stimuli other than DMSO, such as END2 co-culture (Mummery *et al.*, 2007), induction by activin/BMP4 (Gibson *et al.*, 2009) and the 'hanging drop' method of cardiomyocyte induction].

In conclusion, we have demonstrated that Br-DIF-1 but not DIF-1 accelerated the DMSO-induced differentiation of P19CL6 cells into spontaneously beating cardiomyocyte-like cells. Br-DIF-1 reversed the deficit in $Ca_v3.1$ gene expression caused by 1% DMSO. These results suggest that Br-DIF-1 is a useful supplement for accelerating DMSO-induced differentiation of P19CL6 cells into spontaneously beating cardiomyocyte-like cells.

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Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Aggregate formation by P19CL6 cells. (A) Typical image of cultured P19CL6 cells on day 14 (phase-contrast view). (B) Typical image of aggregate formation (arrows) by P19CL6 cells in the presence of 1% DMSO and Br-DIF-1 (3 μM) on day 14 (phase-contrast view).

Figure S2 Br-DIF-1 did not potentiate 1% DMSO-induced differentiation of P19CL6 cells into cardiomyocyte cells. Expression of cardiac specific genes MLC2a (A), MLC2v (B) and ANP (C) in P19CL6 cells on Day 14. Gene expression levels were normalized to G3PDH gene. All ratios were calculated against to the expression level at Day 0. Open bars: control; closed bars: 1% DMSO; hatched bars: Br-DIF-1 (3 μM); and vertically hatched bars: 1% DMSO + Br-DIF-1 (3 μM). Bars represent the mean ± SEM, $n = 4$. *$P < 0.05$ and **$P < 0.01$ compared with control.

Video S1 Typical moving image of spontaneous beating aggregate formed by P19CL6 cells in the presence of 1% DMSO and Br-DIF-1 (3 μM) on Day 14 (phase-contrast view).

Video S2 Typical moving image of spontaneous beating aggregate before adding mibefradil (100 nM, phase-contrast view). The beating rate of this aggregate was 60.3 bpm.

Video S3 Typical moving image of spontaneous beating aggregate 3 h after adding mibefradil (100 nM, phase-contrast view). The beating rate of this aggregate was 5.4 bpm.

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