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# 8-Methyltryptanthrin-Induced Differentiation of P19CL6 Embryonal Carcinoma Cells into Spontaneously Beating Cardiomyocyte-like Cells

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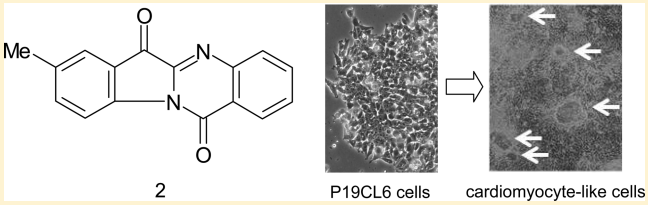
Supporting Information

**ABSTRACT:** Enhancement of cardiac differentiation is critical to stem cell transplantation therapy for severe ischemic heart disease. The aim of this study was to investigate whether several derivatives of tryptanthrin (**1**), extracted from the medicinal plant *Polygonum tinctorium*, induce the differentiation of P19CL6 mouse embryonal carcinoma cells into beating cardiomyocyte-like cells. P19CL6 cells were cultured in α-MEM supplemented with 10% FBS including a test compound or vehicle. Drug-induced differentiation was assessed by measuring the number of beating and nonbeating aggregates and the area of beating aggregates, and the expression of genes involved in cardiac differentiation was evaluated by real-time PCR. A 1 μM concentration of 8-methyltryptanthrin (**2**) induced the differentiation of P19CL6 cells into cardiomyocyte-like cells to a significantly greater degree than 1% dimethyl sulfoxide (DMSO), a conventional differentiation inducer of P19CL6 cells. Furthermore, **2** strongly increased both the number and the area of spontaneously beating aggregates in comparison with DMSO. Two distinct genes of the calcium channel family, Cav1.2 and Cav3.1, underlying cardiac automaticity were significantly expressed in the presence of **2**. Gap junction genes GJA1 and GJA5 contributing to the synchronized contraction of the myocardium were also induced significantly by **2**. These results suggest that **2** successfully differentiated P19CL6 cells into spontaneously beating cardiomyocyte-like cells by activating the gene expression of pacemaker channels and gap junctions.




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 ischemia-reperfusion injury, especially inflammation.[1,2] 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.[3] Furthermore, these drugs provide versatile experimental tools to understand mechanisms that determine cell fate. Exploration of new efficient inducers of cardiomyocyte differentiation is of great importance.[4,5]

The P19CL6 mouse embryonal carcinoma cell line is derived from a teratocarcinoma formed in C3H/He mice.[6] It has been used successfully as a model system for cardiomyocyte differentiation.[7] The P19CL6 cell line has a significant experimental advantage compared to embryonal stem cells. P19CL6 cells can sustain an undifferentiated 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.[6−10] Dimethyl sulfoxide (DMSO) has primarily been used to induce cardiomyocyte differentiation in P19CL6 cells.[7,8] Several effective drugs such as retinoic acid and 5-azacytidine were developed for the effective cardiac myocyte differentiation of P19CL6.[9,10] 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 indoloquinazoline alkaloid tryptanthrin, 6,12-dihydro-6,12-dioxoindolo-(2,1-*b*)-quinazoline) (**1**, Figure 1), was first isolated from the yeast *Candida lipolytica*.[11] It is also contained in a number of plants, *Isatis*,[12] *Strobilanthus*,[13] and *Couroupota* species,[14] which have been used as medicinal herbs for their anti-inflammatory, antipyretic, and analgesic effects. It has been

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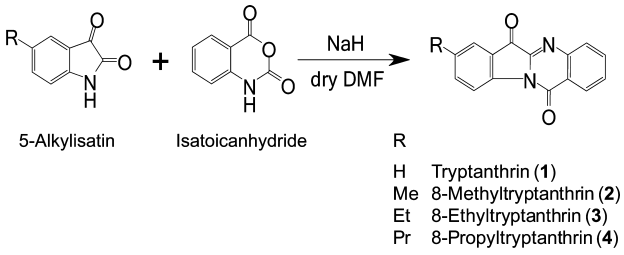


**Figure 1.** Synthesis of tryptanthrin (**1**) and its derivatives (**2**–**4**).

reported to have various biological activities, such as antimicrobial, antitumor, and anti-inflammatory activities.[15] Recently, it was also found that **1** exerts its activity against the murine myelomonocytic leukemia WEHI-3B JCS cell line. Compound **1** induced differentiation of leukemia cells by causing cell cycle arrest and triggering cell differentiation. However, higher concentrations may have killed leukemia cells through apoptosis, possibly through a Fas/caspase-3 pathway.[16,17] Given the differentiation effects of **1** in leukemia 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 verify whether **1** and its derivatives induce cardiomyocyte differentiation in the P19CL6 carcinoma cell line. It is shown here that 8-methyltryptanthrin (**2**), but not **1**, successfully differentiated P19CL6 into spontaneously beating cardiomyocyte-like cells by enhancing the gene expression of pacemaker channels and gap junctions.

## RESULTS AND DISCUSSION

**Effect of 8-Methyltryptanthrin (2) on Induction of Cardiomyocyte Differentiation from P19CL6 Cells.** The effect of cardiomyocyte differentiation from P19CL6 cells of tryptanthrin derivatives was investigated. Cardiomyocyte differentiation was visually confirmed by the formation of aggregates (white arrows in Figure 2A inset). 8-Methyltryptanthrin (**2**) dose-dependently (0.1–1 $\mu$M) induced the formation of aggregates on day 10 and reached a maximal value on day 18 (Figure 2A and E). However, tryptanthrin (**1**), 8-ethyltryptanthrin (**3**), and 8-propyltryptanthrin (**4**) failed to induce aggregate formation. DMSO (1%; positive control) induced the formation of aggregates on day 10 and reached a plateau on day 16 (Figure 2C). The number of aggregates in the presence of **2** (1 $\mu$M) and DMSO on day 18 was 47.3 ± 6.7 and 38.2 ± 8.4 per 8.8 mm$^2$, respectively. The control value (0.1% DMSO alone) was 3.6 ± 1.5 on day 18. Each low concentration (0.1% and 0.3%) of DMSO did not induce cardiomyocyte differentiation from P19CL6 up to day 18 (data not shown).

**Expression of Cardiac-Specific Genes.** The expression of the cardiac-specific genes $\alpha$-myosin heavy chain (MHC), GATA4, and bone morphogenetic protein 2 (BMP2) was investigated to confirm 8-methyltryptanthrin (**2**)-induced cardiac differentiation of P19CL6 cells. DMSO (1%) time-dependently accelerated the expression of these genes (2106.3 ± 866.5, 28.1 ± 4.2, and 83.3 ± 6.8, respectively) on day 18. The control value of each gene in the absence of 1% DMSO was 47.5 ± 41.1, 0.7 ± 0.1, and 2.6 ± 0.6, respectively. **2** (1 $\mu$M) alone also time-dependently and equivalently increased the gene expression of $\alpha$-MHC, GATA4, and BMP2 (1130.1 ± 234.0, 30.3 ± 4.3, and 84.4 ± 5.8, respectively) on day 18 (Figure 3A–C). A low concentration (0.1%) of DMSO and

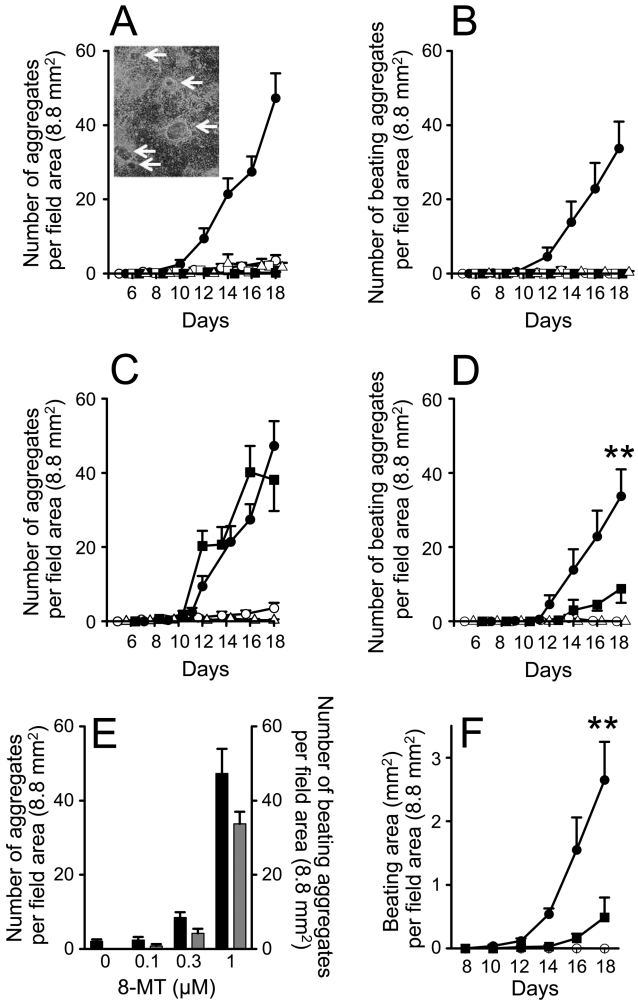


**Figure 2.** Only 8-methyltryptanthrin (**2**) potentiates the cardiomyocyte differentiation of P19CL6 cells. Time-course of changes in the number of aggregates, an index of cardiomyocyte differentiation in P19CL6 cells (A), and the number of spontaneously beating aggregates, an index of differentiation from P19CL6 cells into spontaneously beating cardiomyocyte-like cells (B) induced by tryptanthrin and its derivatives. Open circles: control; solid circles: 8-methyltryptanthrin (**2**, 1 $\mu$M); open squares: tryptanthrin (**1**, 1 $\mu$M); solid squares: 8-ethyltryptanthrin (**3**, 1 $\mu$M); open triangles: 8-propyltryptanthirin (**4**, 1 $\mu$M). Values are mean ± SEM, $n$ = 7. Time-course of changes in the number of aggregates (C) and spontaneously beating aggregates (D) produced by 0.1% DMSO (open triangles), 1% DMSO (solid squares), and **2** (1 $\mu$M, solid circles). No treatment control is represented as an open circle. Values are mean ± SEM, $n$ = 7. **$p$ < 0.01 compared with 1% DMSO on day 18. (E) Dose-dependent increase in the number of aggregates (solid bars) and spontaneously beating aggregates (gray bars) by **2** (0.1–1 $\mu$M) on day 18. Values are mean ± SEM, $n$ = 7. (F) Time-course of changes in the area of spontaneously beating aggregates, an index of the development of synchronized contraction. Open circles: control; solid circles: **2** (1 $\mu$M); solid squares: 1% DMSO. Values are mean ± SEM, $n$ = 9. **$p$ < 0.01 compared with 1% DMSO on day 18. The inset image in A shows differentiated cardiomyocyte-like cells forming aggregates (indicated by white arrows). Some of these aggregates were further differentiated into beating aggregates, namely, spontaneously beating cardiomyocyte-like cells.

tryptanthrin (**1**) failed to induce the expression of these cardiac-specific genes. These results suggest that **2** is an inducer


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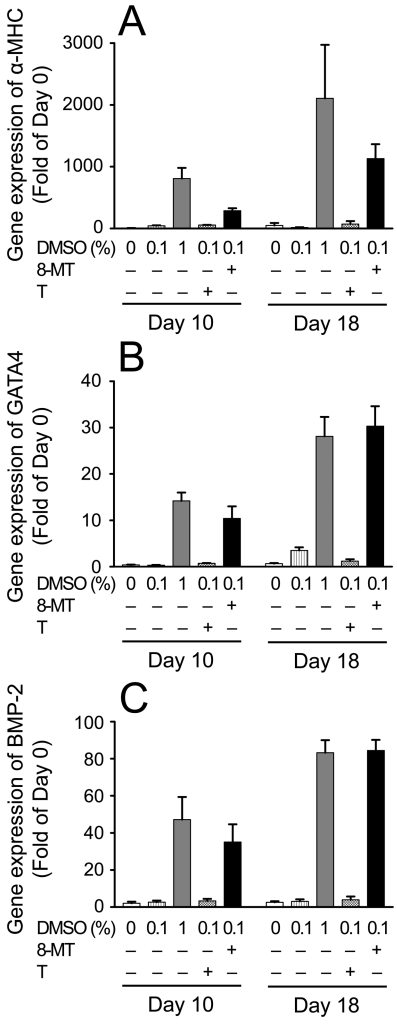


**Figure 3.** 8-Methyltryptanthrin (**2**) potentiates the differentiation of P19CL6 cells into cardiomyocyte-like cells to a similar degree to 1% DMSO. Time-course expression of cardiac-specific genes $\alpha$-MHC (A), GATA4 (B), and BMP2 (C) on day 10 and 18. Gene expression levels were normalized to G3PDH. All ratios were relative to day 0. Open bars: negative control; gray bars: 1% DMSO; solid bars: **2** (1 $\mu$M); vertically hatched bars: 0.1% DMSO; diagonally hatched bars: **1** (1 $\mu$M). Bars represent the mean ± SEM, $n$ = 4.

of differentiation of P19CL6 cells into cardiomyocyte-like cells like 1% DMSO.

**8-Methyltryptanthrin (2)-Induced Differentiation of P19CL6 Cells into Spontaneously Beating Cardiomyocyte-like Cells.** The effect of **2** and other tryptanthrin derivatives on spontaneous beating cardiomyocyte differentiation was investigated using P19CL6 cells. When P19CL6 cells differentiate into spontaneously beating cardiomyocyte-like cells, beating aggregates are produced.[18] DMSO (1%) induced the formation of spontaneously beating aggregates on day 10, and the average number of spontaneously beating aggregates was 8.8 ± 3.8 per 8.8 mm$^2$ on day 18, compared to zero in control cells (Figure 2D). Compound **2** (0.1−1 $\mu$M) induced in a dose-dependent fashion the formation of beating aggregates on day 10 and reached a maximal value on day 18 (Figure 2B and E). The number of spontaneously beating aggregates in the presence of **2** (1 $\mu$M) on day 18 was 33.7 ± 7.2 per 8.8 mm$^2$ ($p$ < 0.01 vs 1% DMSO). The beating rate of cardiomyocyte-like cells in the presence of **2** (1 $\mu$M) was 52.1 ± 5.2 beats per minute (bpm, $n$ = 9) on day 18, which was not significant compared to that in the presence of 1% DMSO alone (45.2 ± 4.2 bpm, $n$ = 9).[18] Compounds **1**, **3**, and **4** did not produce spontaneously beating aggregates. The possibility that compound **2** requires the presence of 0.1% DMSO to induce cardiomyocyte differentiation of P19CL6 cells was excluded because **2** dissolved in 0.03% DMSO by sonication treatment also induced the same extent of cardiomyocyte differentiation of P19CL6 cells (data not shown). These results suggest that **2** is a more potent inducer of differentiation of P19CL6 cells into spontaneously beating cardiomyocyte-like cells than 1% DMSO.

**Gene Expression of Pacemaker Channels.** Spontaneously beating pacemaker cells such as sinoatrial nodal cells express certain characteristic genes such as those for L-type (Cav1.2 and Cav1.3) and T-type (Cav3.1 and Cav3.2) voltage-dependent calcium channels and hyperpolarization-activated cation channels (HCN2 and HCN4). Therefore, the association of the expression of these genes with the production of spontaneously beating aggregates as a result of treatment of P19CL6 cells with either **2** or 1% DMSO was investigated.

Compared with 0.1% DMSO, compound **2** significantly ($p$ < 0.05) increased the expression of Cav1.2 in a time- and concentration-dependent manner. The fold increase in Cav1.2 expression relative to negative control induced by **2** was 1.90 ± 0.22 and 3.38 ± 0.20 on day 10 and 18, respectively, whereas 1% DMSO increased Cav1.2 by only 0.90 ± 0.33- and 2.05 ± 0.16-fold on day 10 and 18, respectively (Figure 4A). On the other hand, DMSO decreased the expression of the other L-type $Ca^{2+}$ channel, Cav1.3, and the expression level was maintained until day 18 (Figure 4B). A similar effect was observed in the presence of **2**. In the case of T-type $Ca^{2+}$ channel, Cav3.1, the expression was strongly decreased in the presence of 1% DMSO (0.11 ± 0.01-fold) on day 10. However, the decrease of this gene expression by **2** was significantly smaller than that by 1% DMSO (0.21 ± 0.02 fold, $p$ < 0.05) (Figure 4C). On day 18, the expression of Cav3.1 induced by **2** and DMSO was not significantly different. The expressions of Cav3.2, HCN2, and HCN4 genes were not altered by **2** (1 $\mu$M). These results suggested that **2**-induced differentiation into spontaneously beating cardiomyocyte-like cells of P19CL6 occurred by significant expression of Cav1.2, an L-type $Ca^{2+}$ channel, and Cav3.1, a T-type $Ca^{2+}$ channel gene.

Two ion channels have been shown to contribute to the pacemaker function of cardiac sinoatrial node cells: $I_{Ca,T}$ and hyperpolarization-activated cation channel (HCN (If)).[4,9,19] In this study, it was confirmed that **2** regulates the expression of the Cav3.1 T-type calcium channel gene, but not the HCN gene. Although **2** also effects expression of the L-type $Ca^{2+}$ channel Cav1.2, this channel relates 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 and Br-DIF-1.[9,18] These results therefore suggest that T-type $Ca^{2+}$ channels have one key role in the spontaneous beating of cardiomyocyte-like cells differentiated from P19CL6. In future work, it will be necessary to demonstrate in more detail the mechanism by which **2** helps the cell to decrease the functional deficit of Cav3.1.

No other tryptanthrin derivatives were able to induce cardiac differentiation. Unlike **2**, the expression of both cardiac-specific genes and the Cav1.2 gene of the P19CL6 cell were not increased by tryptanthrin (**1**) and its alkyl derivatives, **3** and **4**


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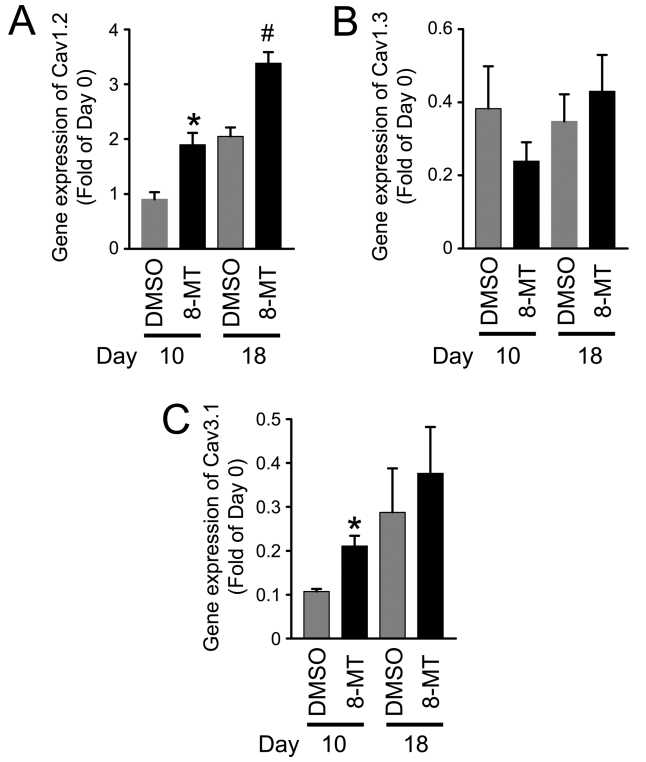


**Figure 4.** 8-Methyltryptanthrin (**2**) potentiated the gene expression of pacemaker channels. Time-course expression of L-type calcium channel genes Cav1.2 (A) and Cav1.3 (B) and pacemaker channel gene Cav3.1 (C) on days 10 and 18. Gene expression levels were normalized to G3PDH. All ratios are expressed relative to day 0. Gray bars: 1% DMSO; solid bars: **2** (1 $\mu$M). Bars represent the mean ± SEM, $n = 6$. $^{*}p < 0.05$ compared with 1% DMSO on day 10, $^{\#}p < 0.05$ compared with 1% DMSO on day 18.

(data not shown). Thus, insufficient expression of these genes may prohibit the differentiation of P19CL6 cells into spontaneously beating cardiomyocyte-like cells. However, these observations cannot explain why **2**, but not **1**, **3**, or **4**, is able to induce the cardiomyocyte differentiation of P19CL6 cells. Structural differentiation between **2** and other tryptanthrin derivatives consists only of functional groups at the 8-position. It is well known that steric differences between methyl and ethyl/propyl groups can affect the reactivity of proteins and nucleotides.[20] In this investigation, it is considered that the methyl group of tryptanthrin derivatives at the 8-position may affect the expression of cardiac-specific genes and Cav1.2. Further study of the role of the methyl group at the 8-position of **2** in the stimulation of Cav1.2 gene expression is needed to clarify the differentiation mechanism of P19CL6 cells.

**8-Methyltryptanthrin (2) Accelerated the Extension of Spontaneously Beating Area of Cardiomyocyte-like Cells.** The area of beating aggregates was measured as an index of cardiac synchronized contraction of myocardium. DMSO (1%) extended the area of beating aggregates to 0.5 ± 0.3 mm$^2$ per field area (8.8 mm$^2$), while **2** (1 $\mu$M, 2.7 ± 0.6 mm$^2$, $p < 0.01$ vs 1% DMSO) significantly accelerated the extension of this area on day 18 (Figure 2F). These phenomena suggested that **2** can accelerate the differentiation of the cardiac conduction system.

**Gene Expression of Gap Junctions.** It is well known that characteristic gap junction proteins such as connexin 43 (GJA1), connexin 40 (GJA5), connexin 45 (GJC1), and connexin 32 (GJB1) contribute to the synchronized contraction of myocardium.[21] Therefore, the gene expression of these proteins was investigated in spontaneously beating areas of P19CL6 cells treated with either **2** or 1% DMSO.

Expression of GJA1 was significantly increased in the presence of **2** in comparison with 1% DMSO (1.38 ± 0.24 and 0.88 ± 0.15, respectively) on day 18 relative to day 0 (Figure 5A). On the other hand, expression of GJA5 in the

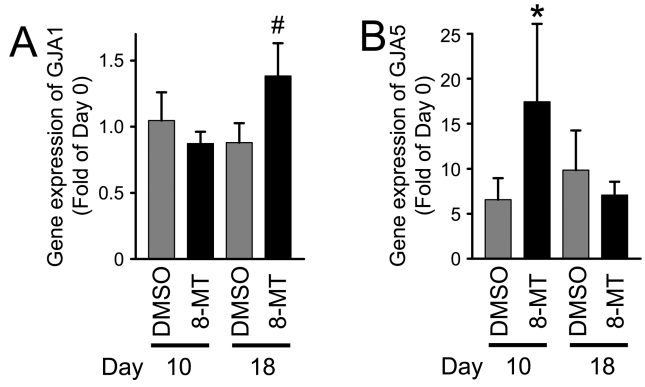


**Figure 5.** 8-Methyltryptanthrin (**2**) accelerated the expression of gap junction genes. Time-course expression of the gap junction genes GJA1 (A) and GJA5 (B) on days 10 and 18. Gene expression levels were normalized to G3PDH. All ratios are relative to day 0. Gray bars: 1% DMSO; solid bars: **2** (8-MT, 1 $\mu$M). Bars represent the mean ± SEM, $n = 4$. $^{*}p < 0.05$ compared with 1% DMSO on day 10, $^{\#}p < 0.05$ compared with 1% DMSO on day 18.

presence of **2** (17.44 ± 8.66-fold) was significantly increased compared to that in the presence of 1% DMSO (6.56 ± 2.40-fold) on day 10 relative to day 0 (Figure 5B). This expression of GJA5 was equal between **2** and DMSO up to day 18 (7.06 ± 1.47- and 9.84 ± 4.41-fold, respectively). The expression of GJA4, GJB1, and GJC1 genes was not altered by **2** (1 $\mu$M).

GJA1 is well known as the major protein of gap junctions in the heart and are thought to have a crucial role in the synchronized contraction of the heart and during embryonal development.[22] However, the expression of GJA1 was significantly but weakly increased on day 18. On the other hand, GJA5 is known to specifically localize in the right atrium, right ventricle, and atrium-ventricular node and His band.[23] In this study, it was strongly expressed in the presence of **2** on day 10. From these results, GJA5 is thought to especially contribute to the synchronization of cardiomyocyte-like cells differentiated from P19CL6 cells.

DMSO has been used to induce differentiation in a variety of human mesenchymal stem cells.[24,25] Our present study has demonstrated the possibility that **2** is also a more effective differentiation inducer of P19CL6 cells into spontaneously beating cardiomyocyte-like cells in comparison to 1% DMSO. For the clinical application of **2**, further investigation using other human stem cell lines will be necessary.

In conclusion, as depicted in Figure 6, it is demonstrated that, in comparison with 1% DMSO, 8-methyltryptanthrin (**2**) differentiated P19CL6 cells into spontaneously beating cardiomyocyte-like cells, namely, pacemaker-like cells, by inducing the expression of two calcium channel genes, Cav1.2 and Cav3.1. Compound **2** also widely develops the area of these cells, resulting in synchronized contraction of myocardium, by increasing the expression of two gap junction genes, GJA1 and GJA5. These results suggest that **2** is a useful tool for


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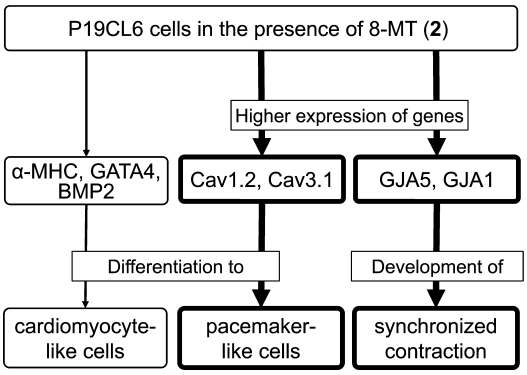


**Figure 6.** Possible mechanism of 8-methytryptanthrin (**2**)-induced differentiation of P19CL6 cells into spontaneously beating cardiomyocyte-like cells. As indicated by narrow arrows, 8-methyltryptanthrin (**2**) differentiated P19CL6 cells into cardiomyocyte-like cells. Compound **2** induces cardiac-specific gene expression such as α-MHC, GATA4, and BMP2. The degree of expression was equal to that of the positive control, 1% DMSO. In comparison with 1% DMSO, **2** significantly upregulates the expression of two calcium channel genes, Cav1.2 and Cav3.1, which may induce differentiation of P19CL6 cells into spontaneously beating cardiomyocyte-like, i.e., pacemaker-like, cells. Treatment of P19CL6 cells with **2** also widely developed the area of these beating cells, resulting in synchronized contraction of myocardium, due to higher expression of two gap junction genes, GJA1 and GJA5. Bold arrows indicate these pathways.

accelerating the differentiation of P19CL6 cells into spontaneously beating cardiomyocyte-like cells. It is hoped that these results will help to further clarify the mechanism of 8-methyltryptanthrin-induced cardiomyocyte differentiation of P19CL6 cells and develop new therapies for myocardial infarction and heart failure.

## EXPERIMENTAL SECTION

**Materials.** Penicillin, α-minimal essential medium (α-MEM), and streptomycin were purchased from Invitrogen (Grand Island, NY, 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. 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.

**Synthesis of Tryptanthrin (1) and Its Derivatives.** Tryptanthrin (**1**) was isolated from *Polygonum tinctorium* W. T. Aiton (Polygonaceae) using methods described previously.[26] As outlined in Figure 1, tryptanthrin (**1**) and its derivatives (**2**, **3**, and **4**) were prepared by condensation of isatoican hydride with isatin or corresponding 5-alkylisatins according to the general procedure.[27] Synthesis of 8-methyltryptanthrin (**2**), which is a typical example, was performed as follows. Under a flow of dry nitrogen, 0.66 g (4.1 mmol) of 5-methylisatin in 30 mL of dry dimethylformamide (DMF) was added over a 15 min period to 0.097 g (4.0 mmol) of NaH with stirring. To the resulting deep purple liquid was added 0.67 g (4.1 mmol) of isatoicanhydride in 30 mL of dry DMF with ice cooling over a 30 min period. The reaction mixture was stirred overnight at room temperature and then quenched with 0.9 mL of methanol. The resulting mixture was diluted with 120 mL of chloroform and washed once with water. The aqueous layer was extracted three times with chloroform, and the combined organic layers were dried (anhydrous sodium sulfate) and concentrated. Crystallization from chloroform–ethyl acetate (2:1) afforded the pure compound (0.60 g) in 56% yield. The structures of these compounds were determined by fast-atom bombardment MS, electrospray ionization MS, 500 MHz $^1$H NMR, and 125 MHz $^{13}$C NMR spectra. The mass spectra showed the corresponding molecular ion peaks, and the chemical shifts and the integral ratios of protons were appropriate in the $^1$H NMR spectra. The physical data for **1** and **2** were exactly identical with the literature values.[27−30] The purity of these compounds (**1**−**4**) used for experiments was more than 98%, as assessed by $^1$H NMR, $^{13}$C NMR, HPLC, and HRESIMS.

*8-Ethyltryptanthrin (**3**):* yellow solid; mp 218−219 °C; $^1$H NMR ($CDCl_3$, 500 MHz) δ 8.52 (1H, d, *J* = 8.5 Hz, H-10), 8.44 (1H, dd, *J* = 8.0, 1.5 Hz, H-1), 8.04 (1H, d, *J* = 8.0 Hz, H-4), 7.84 (1H, ddd, *J* = 8.0, 8.0, 1.5 Hz, H-3), 7.75 (1H, d, *J* = 2.0 Hz, H-7), 7.67 (1H, ddd, *J* = 8.0, 8.0, 1.0 Hz, H-2), 7.61 (1H, dd, *J* = 8.5, 2.0 Hz, H-9), 2.76 (2H, q, *J* = 7.5 Hz, $C\underline{H}_2CH_3$), 1.30 (3H, t, *J* = 7.5 Hz, $CH_2C\underline{H}_3$); $^{13}$C NMR ($CDCl_3$, 125 MHz) δ 183.0 ($\underline{C}$=O), 158.2 ($\underline{C}$=O), 146.9, 144.9, 144.7, 144.0, 138.3, 135.3, 130.9, 130.4, 127.7, 124.6, 124.0, 122.3, 118.0 (carbons of tryptanthrin ring), 28.7 ($\underline{C}H_2CH_3$), 15.5 ($CH_2\underline{C}H_3$); HRESIMS *m*/*z* 277.1018 $[M + H]^+$ (calcd for $C_{17}H_{13}N_2O_2$, 277.0977).

*8-Propyltryptanthrin (**4**):* yellow solid; mp 202−203 °C; $^1$H NMR ($CDCl_3$, 500 MHz) δ 8.51 (1H, d, *J* = 8.5 Hz, H-10), 8.44 (1H, dd, *J* = 8.0, 1.5 Hz, H-1), 8.03 (1H, d, *J* = 8.0 Hz, H-4), 7.85 (1H, ddd, *J* = 8.0, 8.0, 1.5 Hz, H-3), 7.72 (1H, d, *J* = 2.0 Hz, H-7), 7.67 (1H, ddd, *J* = 8.0, 8.0, 1.0 Hz, H-2), 7.59 (1H, dd, *J* = 8.5, 2.0 Hz, H-9), 2.69 (2H, q, *J* = 7.5 Hz, $C\underline{H}_2CH_2CH_3$), 1.70 (2H, m, $CH_2C\underline{H}_2CH_3$), 0.95 (3H, t, *J* = 7.5, $CH_2CH_2C\underline{H}_3$); $^{13}$C NMR ($CDCl_3$, 125 MHz) δ 183.1 ($\underline{C}$=O), 158.2 ($\underline{C}$=O), 146.9, 144.9, 144.7, 142.6, 138.8, 135.2, 131.0, 130.4, 127.7, 125.2, 124.0, 122.3, 118.0 (carbons of tryptanthrin ring), 37.7 ($\underline{C}H_2CH_2CH_3$), 24.4 ($CH_2\underline{C}H_2CH_3$), 13.9 ($CH_2CH_2\underline{C}H_3$); HRESIMS *m*/*z* 291.1151 $[M + H]^+$ (calcd for $C_{18}H_{15}N_2O_2$, 291.1134).

**Culture and Cardiomyocyte Differentiation of P19CL6 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 penicillin, and 100 μg/mL streptomycin. All cells were passaged at 48 h intervals. To induce differentiation, the cells were cultured at a density of $2.0 \times 10^4$ cells per 35 mm dish in medium containing 1% DMSO as a positive control and tryptanthrin derivatives. 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).

Aggregates formed after 10 days of culture and subsequently increased time-dependently (Figure 2A inset). Numbers of aggregates were measured (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.[18,31] Images (×100) from five separate visual fields (total 8.8 $mm^2$) were obtained from each inset, and the number of aggregates was evaluated. The area of beating aggregates (as an index of development of synchronized contraction) was calculated using Adobe Photoshop software. These measurements were performed only until 18 days because, in this experimental condition, extending incubation beyond 18 days resulted in degeneration of aggregates and loss of aggregate rhythmicity.

**Measurement of Gene Expression.** Total RNA was isolated from cells using 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


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**Table 1. Primers Used for Quantitative Real-Time PCR**

| gene symbol | GenBank accession no. | sequences (5′−3′) |
|---|---|---|
| G3PDH | AF106 860 | forward: tgcaccaccaactgcttag |
| | | reverse: ggatgcagggatgatgttc |
| α-MHC | NM_010 856 | forward: catggctacactcttctctacctatgc |
| | | reverse: gagcagacactgtttggaagga |
| GATA4 | NM_008 092 | forward: ccaatttgggattttcttttcc |
| | | reverse: ccaactcgctcaaaatatatacgaatta |
| BMP2 | NM_007 553 | forward: acacagggacacaccaaccat |
| | | reverse: tgtgaccagctgtgttcatcttg |
| Cav1.2 | AB259 049 | forward: gagccacggtgaatcagga |
| | | reverse: gcagtactcggcttcttcactca |
| Cav1.3 | AJ437 292 | forward: tacgtggtgaactcctcgcctttcgaat |
| | | reverse: ccaggcagagcgtgttga |
| Cav3.1 | AJ012 569 | forward: cctgagaatttcagcctccc |
| | | reverse: gatcgcatgccgttctcc |
| Cav3.2 | NM_021 415 | forward: atgtactcactggctgtgacc |
| | | reverse: gagtccaaaagagtgtgggc |
| HCN2 | NM_008 226 | forward: cgaggtgctggaggaatacc |
| | | reverse: tgcgatctagccggtcaatag |
| HCN4 | AF064 874 | forward: cggcaagaagatgtactttatcca |
| | | reverse: ttggtctctttgttgcccttagt |
| GJA1 | BC055 375 | forward: aattcctcctgccgcaattac |
| | | reverse: cctgccccattcgattttg |
| GJA4 | BC056 613 | forward: catcttcatgctggtggtagga |
| | | reverse: ctttatctcccggctgacaca |
| GJA5 | BC053 054 | forward: acctatgtctgcaccattctgatc |
| | | reverse: catgcagggtatccaggaaga |
| GJB1 | BC026 833 | forward: tgcaacagcgtctgctatgac |
| | | reverse: ctgttggtgagctacgtgcatt |
| GJC1 | NM_011 593 | forward: tgggtaacaggagttctggtgaa |
| | | reverse: atggttgtggatctcctctagca |

expression was quantified as the fold change in expression of each gene relative to expression on day 0.

**Statistical Analysis.** All statistical analyses were carried out using 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.

## ■ ASSOCIATED CONTENT

### Supporting Information

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### Notes

The authors declare no competing financial interest.

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