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Hedgehog/GLI-mediated transcriptional inhibitors from *Zizyphus cambodiana*

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ABSTRACT

The aberrant hedgehog (Hh)/GLI signaling pathway causes the formation and progression of a variety of tumors. By screening tropical plant extracts by using our screening system, *Zizyphus cambodiana* was found to include Hh/GLI signaling inhibitors. Bioassay-guided fractionation of this plant extract led to the isolation of three active pentacyclic triterpenes, colubrinic acid (**1**), betulinic acid (**2**) and alphitolic acid (**3**), as potent inhibitors. The inhibition of GLI-related protein expression with **1** or **2** was observed in HaCaT cells with exogenous GLI1, or human pancreatic cancer cells (PANC1), which express Hh/GLI components aberrantly. The expressions of GLI-related proteins PTCH and BCL2 were clearly inhibited by **1** or **2**. We also examined the cytotoxicity of these active compounds against PANC1, human prostate cancer cells (DU145) and mouse embryo fibroblast cells (C3H10T1/2). The cytotoxicity against cancer cells (PANC1 and DU145) by **1** or **2** would be caused by inhibition of the expression of the anti-apoptosis protein BCL2. These pentacyclic triterpene inhibitors showed an important relationship between Hh/GLI signaling inhibition, the decrease of BCL2, and cytotoxicity against cancer cells.

1. Introduction

The hedgehog (Hh)/GLI signaling pathway has been implicated not only in a variety of developmental processes of differentiation and proliferation in a wide range of organisms,[1] but also in the formation and development of different tumors,[2,3] including the skin,[4] brain,[5] prostate,[6] digestive tract,[7] pancreas,[8] and lung.[9] A Hh ligand is secreted into the cytosol from Hh-sending cells. On Hh-receiving cells, patched (PTCH) receptor, a 12-pass transmembrane protein, interacts with the Hh ligand and smoothened (SMO), a 7-pass transmembrane protein. After the Hh ligand binds to PTCH, signal transduction is activated, resulting in the release of the transcriptional factor GLI from a macromolecular complex on microtubules. The released GLI is located in the nucleus to regulate several gene expressions. In some types of human tumors, Hh/GLI signaling is constitutively activated because of mutations in PTCH or SMO, leading to tumor formation and progression. Thus, targeted Hh/GLI signaling is anticipated as an effective cancer therapeutic strategy.[10] Cyclopamine has been identified as an inhibitor of Hh signaling by binding to SMO,[11,12] and other types of SMO antagonists have been reported such as CUR61414[13] and SANTs.[14] In addition, several small-molecule inhibitors of Hh signaling inhibitors including GLI-mediated transcription inhibitors (GANTs)[15] and inhibitor of class IV alcohol dehydrogenase (JK184)[16] have been reported. But there is still an urgent need to identify different types of GLI-mediated transcriptional inhibitors.

To identify inhibitors of the Hh/GLI signaling pathway from natural resources, we have recently reported the successful construction of a cell-based screening assay system for the Hh/GLI signaling pathway (Fig. 1).[17] This is an assay using a GLI-dependent luciferase reporter in human keratinocyte cells (HaCaT) expressing GLI1

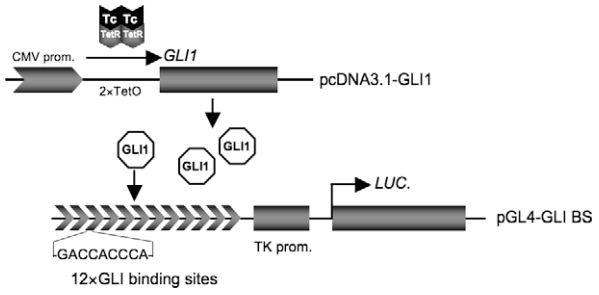

Figure 1. The assay system with T-REx (tetracycline-regulated expression system). pcDAN3.1-GLI1 expresses exogenous GLI1 protein by tetracycline-regulated CMV promoter, and GLI1 binds to GLI-binding site on pGL4-GLI BS. Tetracycline removes TetR to start GLI1 expression. Tc, tetracycline; TetR, tetracycline repressor; TetO, tetracycline operator; CMV, cytomegalovirus promoter; GLI1, transcriptional factor of Hh/GLI signaling pathway.

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under tetracycline control (T-REx system). The 12 consecutive GLI-binding sites (12 × GACCACCCA) and the TK promoter were inserted into the pGL4.20 plasmid (Promega). The constructed plasmid, pGL4-GLI BS, was stably transfected into HaCaT cells expressing exogenous GLI1 protein under tetracycline control. During the screening of natural resource libraries, including plant extracts and actinomycete extracts with the assay system, we identified some natural products and natural plant extracts as GLI1-mediated transcriptional inhibitor samples. Among them, we chose the methanol extract of *Zizyphus cambodiana* (Rhamnaceae) to examine active components, and isolated some GLI-mediated transcriptional inhibitors. Finally, we determined the effect of these inhibitors on protein expression related to the Hh/GLI signaling pathway.

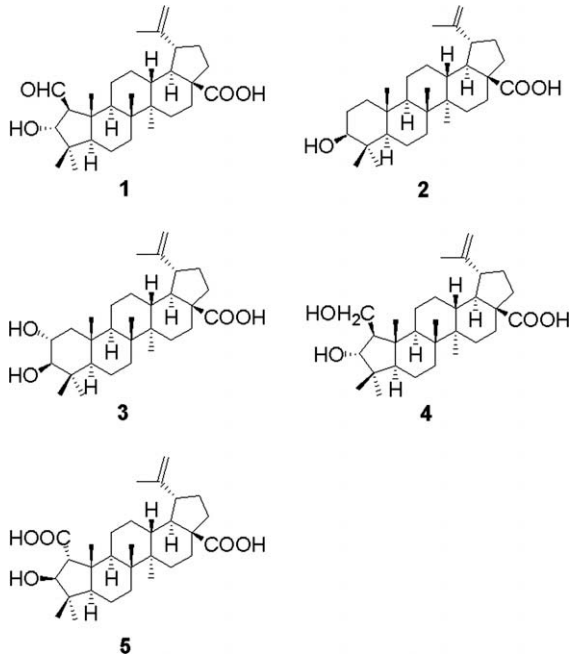

Figure 2. Chemical structures of isolated compounds (**1**–**5**) from *Zizyphus cambodiana*.

2. Results

The methanol extract of *Z. cambodiana* was partitioned between H_2O and *n*-hexane, ethyl acetate. Both *n*-hexane and ethyl acetate-soluble fractions showed GLI1-mediated transcriptional inhibitory activity at 100 μg/mL, and revealed the presence of nearly the same constituents by TLC analysis, so both extracts were combined. The combined extracts was subjected to silica gel, ODS and/or Sephadex LH-20 column chromatography and further purification by reversed-phase HPLC under the guidance of GLI1-mediated transcriptional inhibitory activity. We isolated three active compounds (**1**, **2** and **3**), together with two analogues (**4** and **5**), which have no inhibition activity. These isolated compounds were identified as colubrinic acid (**1**),[18,19] betulinic acid (**2**),[20] alphitolic acid (**3**),[21,22] ceanothanolic acid (**4**),[19] and ceanothic acid (**5**),[23] on the basis of comparisons with their spectral data in the literature (Fig. 2). All of the compounds have the same structure framework, a pentacyclic triterpene, with difference at the A-ring. Colubrinic acid (**1**), betulinic acid (**2**), and alphitolic acid (**3**) dose-dependently inhibited GLI1-mediated transcriptional activity with little effect on cell viability (Fig. 3), and **4** and **5** were inactive even at 100 μM. The IC_{50} values of **1**, **2** and **3** against GLI1-mediated transcriptional inhibitory activity were 38, 32, 42 μM, respectively (Table 1).

We further examined the effects of **1** and **2** on the protein expression related to Hh/GLI-mediated transcription (Fig. 4). First, we confirmed the expression of exogenous GLI1 protein in HaCaT cells under tetracycline control by Western blotting, and sufficient GLI1 protein was expressed even after treatment with each con-

Table 1
IC_{50} values (μM) of identified compounds (**1**–**5**) of GLI1-mediated transcriptional inhibitory activity, and cytotoxicity against PANC1, DU145, and C3H10T1/2 cells

Compounds	GLI1 transcriptional inhibition (IC_{50}: μM)	Cytotoxicity (IC_{50}: μM)		
		PANC1	DU145	C3H10T1/2
1	38	43	78	167
2	32	44	37	82
3	42	41	70	145
4	133	>200	>200	>200
5	>200	195	>200	>200

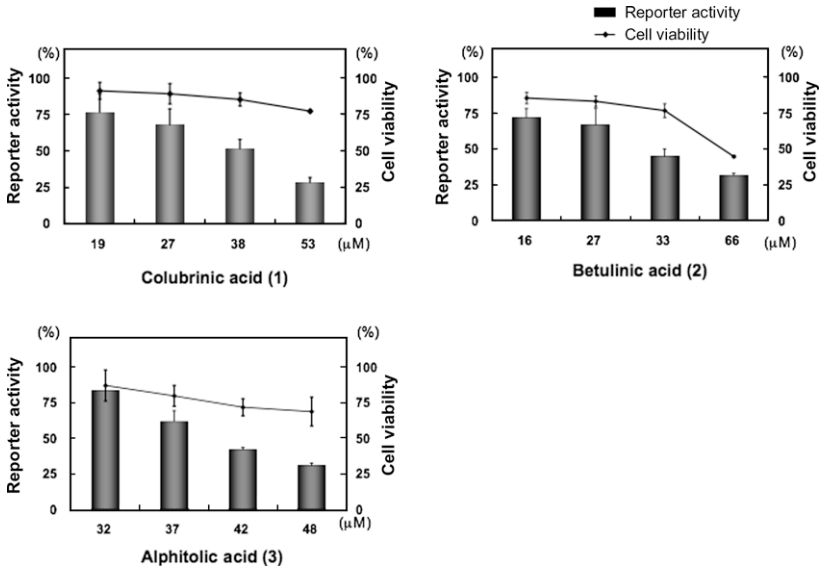

Figure 3. Inhibition of GLI1-mediated transcriptional activity (solid column; reporter activity) in a dose-dependent manner with cell viability (solid curves). Compounds were assayed in triplicate. All compounds were dissolved in DMSO. Bars, s.d.

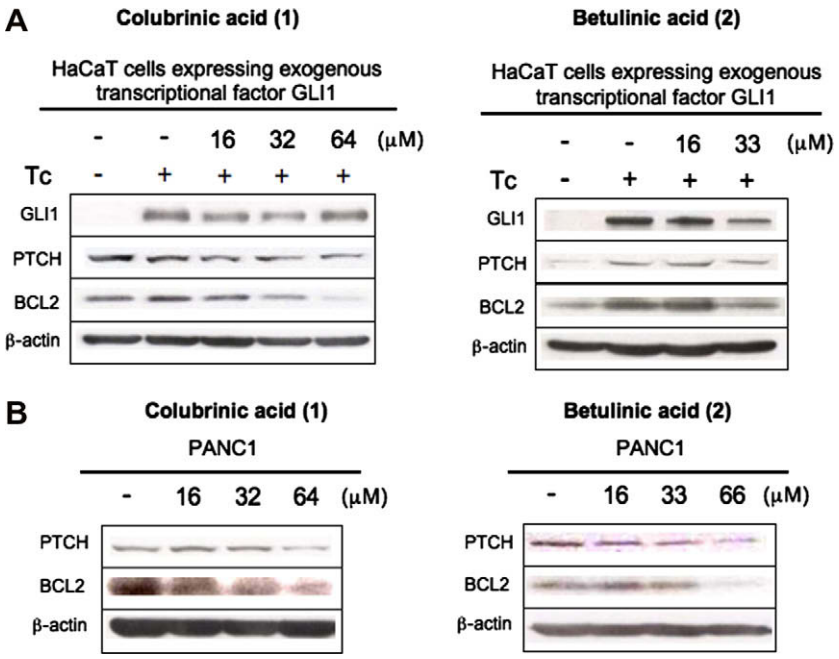

Figure 4. Inhibition of GLI-related proteins with **1** or **2**. (A) PTCH and BCL2 protein levels in GLI1-overexpressing HaCaT cells with treatment with **1** or **2**. (B) PTCH and BCL2 protein levels in PANC1 cells with treatment with **1** or **2**.

centration of **1** at almost the same level (Fig. 4A). Because PTCH and BCL2 expressions are known to depend on GLI-mediated transcription, we investigated the levels of PTCH and BCL2 by Western blotting in GLI1-overexpressing HaCaT cells. Compound **1** showed a dose-dependent reduction of the levels of PTCH and BCL2 proteins. Compound **2** decreased BCL2, PTCH and GLI1 proteins. Because GLI1 was regulated by GLI2, which was the protein affected by Hh signaling, some different mechanism from compound **1** might be included in the case of HaCaT cells. Furthermore, we confirmed the effect of **1** and **2** on the protein expression of PTCH and BCL2 in a human pancreatic cancer cell line (PANC1) (Fig. 4B). The Hh/GLI signaling pathway regulates these protein expressions. Compounds **1** and **2** clearly decreased the expression of PTCH and apoptosis-inhibiting protein BCL2.

PANC1 and DU145 express numerous Hh/GLI signaling pathway components, including sonic hedgehog, PTCH, Suppressor of Fused [Su(Fu)], GLI1, and GLI2, resulting from aberrant Hh/GLI signaling in the cells. Next, we confirmed the cytotoxicity of compounds **1**–**5** against PANC1 and DU145 using a fluorometric microculture cytotoxicity assay (FMCA) (Fig. 5 and Table 1). Compounds **1**, **2** and **3** were cytotoxic against PANC1 cells with IC_{50} values of 43, 44 and 41 μM, respectively. Compounds **1**, **2** and **3** also showed cytotoxicity against DU145 cells with IC_{50} values of 78, 37 and 70 μM, respectively (Table 1). Compounds **4** and **5** were not Hh/GLI signaling inhibitors, supporting the lower cytotoxicity against PANC1 and DU145. A mesenchymal progenitor (C3H10T1/2) cell line derived from the mouse embryonic mesodermal was also examined, because this normal cell is Hh responsive but not reliant

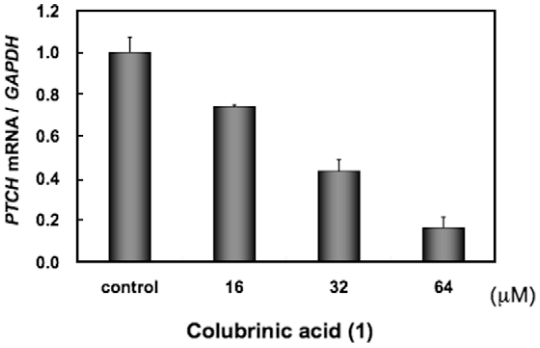

Figure 6. Inhibition of GLI-mediated mRNA expression of *PTCH* by **1** in PANC1 cells. *GAPDH* was used as an internal control. Compounds were assayed in triplicate and dissolved in DMSO. Bars, s.d.

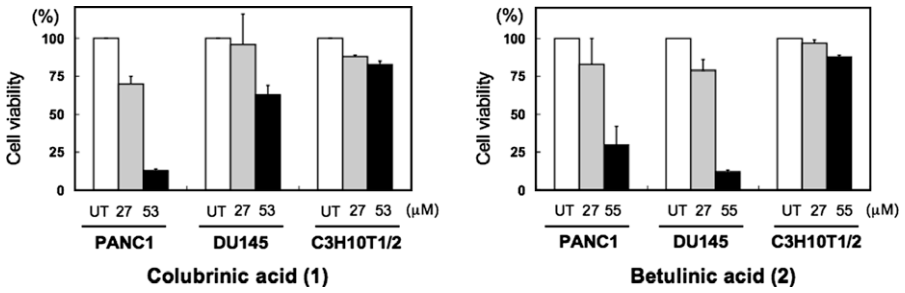

Figure 5. Cytotoxic effects of **1** or **2** on PANC1, DU145, and C3H10T1/2 cells. Compounds were assayed in triplicate. All compounds were dissolved in DMSO. Bars, s.d. UT, compound untreated.

on Hh for survival. Compounds **1**, **2** and **3** showed cytotoxicity only at higher concentrations against C3H10T1/2 than those against PANC1 and DU145 cells (Table 1). As shown in Figure 5, it was obvious that normal cells (C3H10T1/2) were less affected by **1** or **2**. From the clinical point of view, these results are important because these compounds act as anticancer agents without affecting normal cells.

Moreover, we examined inhibition of GLI-mediated mRNA expression by **1**. Colubrinic acid (**1**) inhibited the mRNA production of *PTCH* in PANC1 cells in the dose-dependent manner. This result clearly shows **1** inhibits GLI-mediated transcription (Fig. 6).

3. Discussion

In this study, we identified three pentacyclic triterpenes as potent inhibitors of the Hh/GLI signaling pathway. Betulinic acid (**2**) has been studied as a cytotoxic compound against several caner cell lines,[24] while colubrinic acid (**1**), and alphitolic acid (**3**) have been little reported. In this study, **1**, **2** and **3** showed inhibitory activity in the Hh/GLI signaling pathway. Compound **2** was reported to induce apoptosis by activation of mitochondria,[25–27] to perturb cell cycle progression[25]; however, the precise mechanism is still under investigation. The overexpression of apoptosis-inhibition protein BCL2 may rescue cancer cells from cytotoxic effects of **2**,[28] and **2** induced apoptosis in cancer cells by decreasing the mRNA expression of *Bcl2*[29] and *Cyclin D1*.[30] It has also been reported that the Hh/GLI signaling pathway in cancer cells regulates the expression of *Bcl2* and *Cyclin D1*. Because we demonstrated that **2** acts as a potent inhibitor of the Hh/GLI signaling pathway in this study, this signal inhibition would be one reason for the decrease of *Bcl2* and *Cyclin D1* expression, which causes the death of cancer cells.

In conclusion, we identified three pentacyclic triterpenes (**1**–**3**) as potent Hh/GLI signaling inhibitors. These compounds showed an important relationship among Hh/GLI signaling inhibition, the decreased expression of the anti-apoptosis protein BCL2, and cytotoxicity against cancer cells. These compounds might become good tools and/or leads to new agents in the investigation of Hh/GLI signaling pathway inhibitors.

4. Experimental

4.1. Extraction and separation

The plant *Zizyphus cambodiana* (Rhamnaceae) was collected in Khon Kaen, Thailand. A voucher specimen (6-674) was deposited in the Faculty of Agriculture, Khon Kean University. The leaves (451.4 g) of *Z. cambodiana* were extracted with MeOH. Part (15.5 g/73.7 g) of the MeOH extract was partitioned with H_2O (400 mL) between *n*-hexane (400 mL × 4), and the aqueous phase was further extracted with EtOAc (400 mL × 4), to afford hexane extract (0.48 g), EtOAc extract (3.3 g) and H_2O extract (9.0 g). In the GLI1-mediated transcriptional assay, the hexane and EtOAc extracts were active at 100 μg/mL, and their luciferase activities were 39% and 33%, respectively, with high cell viability (>60%). Since the hexane and EtOAc extracts contained almost the same constituents by TLC analysis, their extracts were combined and used for silica gel column chromatography (Column A: 30 × 470 mm), eluted with gradient mixtures of hexane/EtOAc (9:1–0:1) and EtOAc/MeOH (4:1–0:1) to give 19 fractions (frs 1A–1S). In the assay of frs 1A–1S, frs 1G, 1I, 1J, 1K, 1L, 1M and 1N showed activity at 50 μg/mL with high cell viability (>60%). Fr. 1K (96 mg) of column A was separated by Sephadex LH-20 column chromatography (16 × 690 mm) eluted with $CHCl_3$/MeOH (1:1) to give two fractions. The latter fraction (53.6 mg) was purified with reversed-phase HPLC (Develosil ODS-HG-5, 10 × 250 mm; eluent, H_2O/MeOH (1:4); flow rate, 2.0 mL/min) to afford **1** (5.7 mg, t_R 35 min). Fr. 1I (80 mg) of column A was subjected to Sephadex LH-20 column chromatography (16 × 680 mm) eluted with $CHCl_3$/MeOH (1:1) followed by reverse-phase HPLC (Develosil ODS-HG-5, 10 × 250 mm; eluent, H_2O/MeOH (3:17); flow rate, 2.0 mL/min) to afford **2** (3.1 mg, t_R 41 min). Fr. 1L (246 mg) of column A was separated with Sephadex LH-20 column chromatography (Column B; 16 × 680 mm) eluted with $CHCl_3$/MeOH (1:1) to give five fractions. The last fraction was purified by preparative TLC to afford **5** (169 mg, TLC on a silica gel plate; eluent, $CHCl_3$/MeOH (4:1); R_f 0.57). The fourth fraction (15 mg/27 mg) of column B was purified by reverse-phase HPLC (Develosil ODS-HG-5, 10 × 250 mm; eluent, H_2O/MeOH (1:3); flow rate, 2.0 mL/min; UV detection at 254 nm) to afford **1** (2.8 mg, t_R 58 min) and **4** (2.6 mg, t_R 77 min). Fr. 1O (69.9 mg), which was a non-active fraction, from column A was separated by silica gel chromatography (16 × 400 mm) eluted with a gradient system of $CHCl_3$/MeOH (50:1–10:1), followed by a reverse-phased HPLC (Mightysil RP-18 GP, 20 × 250 mm; eluent, H_2O/MeOH (3:17); flow rate, 5.0 mL/min) of the second fraction (40.4 mg) to give **3** (1.9 mg, t_R 43 min).

4.2. Sample screening: GLI1-mediated transcriptional activity assay

In the cell-based assay system, three plasmids (pcDNA6/TR, pcDNA3.1-GLI1, and pGL4-GLI-luc) are stably co-transfected in HaCaT cells. pcDNA3.1-GLI1 plasmid produces exogenous GLI1 proteins, and pGL4-GLI-luc plasmid is a reporter construct which produces luciferase by GLI1-mediated transactivation. This assay is based on 'Tetracycline-Regulated Expression system (Tet-On system)', and the expression of GLI1 is regulated by a tetracycline repressor. The addition of tetracycline produces an amount of exogenous GLI1 proteins in the assay cell, and subsequently luciferase expression is increased.

Cells (HaCaT-GLI1-luc) were cultured in a 96-well white plate at 2×10^4 cells per well at 37 °C for 12 h. After incubation, 1 μg/mL of tetracycline was added to each well to express exogenous GLI1 protein, and cells were incubated at 37 °C for 12 h. The medium was replaced with tetracycline-free medium containing different concentrations of each sample. After treatment at 37 °C for 12 h, luciferase activity was measured in a microplate luminometer (Thermo) using the Bright-Glo™ Luciferase Assay System (Promega) according to the manufacturer's protocol. The ratio of the reporter luciferase activity of sample-treated cells/non-treated cells was calculated as GLI1-mediated transcriptional activity. At the same time, the cell viability of the sample-treated cells was measured. The same cells (HaCaT-GLI1-luc) were seeded onto a 96-well black plate at 2×10^4 cells per well at 37 °C for 24 h. Different concentrations of samples were added at the same time as the luciferase assay, and the cells were incubated at 37 °C for 12 h. Cell viability was determined by fluorometric microculture cytotoxicity assay (FMCA) using a fluorescence plate reader (Thermo). The ratio of sample-treated cells/non-treated cells was calculated as cell viability.

4.3. Western blotting analysis

Tetracycline-regulated HaCaT cells (exogenous GLI1 expression) were seeded onto a 10 cm dish at 2×10^6 cells, and pre-incubated at 37 °C for 12 h. To express exogenous GLI1 protein, 1 μg/mL of tetracycline was added, and cells were incubated at 37 °C for 12 h. The medium was then removed and different concentrations of compounds (**1** and **2**) diluted with tetracycline-free medium were added. After 24 h treatment of compounds, cells were washed with PBS and then homogenized in lysis buffer (20 mM

Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 10 mM EDTA, 1 mM sodium orthovanadate, and 0.1 mM NaF) containing 1% proteasome inhibitor cocktail (Nacalai Tesque, Tokyo, Japan), and incubated on ice for 30 min. The cell lysate were centrifuged for 30 min at 4 °C, and the supernatants were resolved by electrophoresis on a 7.5% and 12.5% polyacrylamide gel and transferred to a polyvinylidine difluoride (PVDF) membrane (Bio-Rad). After blocking with TBST (10 mM Tris–HCl, pH 7.4, 100 mM NaCl, and 0.1% Tween 20) containing 5% skimmed milk for 1 h at room temperature, the blots were hybridized at room temperature for 1 h with primary antibodies. β-Actin was used as an internal control. After washing with TBST, the blots were incubated at room temperature for 1 h with secondary antibodies conjugated with horseradish peroxidase. After washing, the immunocomplexes were visualized using an ECL Advance Western detection system (GE Healthcare/Amersham Biosciences) or Immobilon Western (Millipore). PTCH and BCL2 expression in PANC1 was determined by the same method. The analysis was performed using antibodies to GLI1 (1:200), PTCH (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and BCL2 (1:4000) (Sigma) as primary antibodies, and anti-goat IgG (Sigma), anti-rabbit IgG and anti-mouse IgG (Amersham Biosciences) as secondary antibodies.

4.4. Cytotoxicity test

PANC1 and C3H10T1/2 were from RIKEN BRC. DU145 were from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. Cells (PANC1, DU145 and C3H10T1/2) were seeded onto a 96-well black plate at 1×10^4 cells per well, and pre-incubated at 37 °C for 24 h. The medium was replaced with fresh medium containing different concentrations of each compound, and the cells were incubated at 37 °C for 24 h. After the medium was removed, cell proliferation was determined by fluorometric microculture cytotoxicity assay (FMCA) using a fluorescence plate reader (Thermo). The ratio of living cells was determined as the fluorescence in the sample wells expressed as a percentage of that in the control cells, and cytotoxic activity was indicated as an IC_{50} value.[31]

4.5. RNA isolation and real time RT-PCR analysis

Total RNA was isolated from cells that were treated with compound **1** using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized and RT-PCR reactions were performed using the RT-PCR SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's instructions. The reactions were performed in Mx3000P QPCR System (Stratagene), using the following RT-PCR program: 50 °C for 2 min (initial incubation), 95 °C for 2 min (initial denaturation), and then 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 30 s (annealing, extension). Sequences for the primers were as follows: *PTCH*; 5′-TCCTCGTGTGCGCTGTCTTCCTTC-3′ and 5′-CGTCAG AAAGGCCAAAGCAACGTGA-3′ (200 bp product), and human *GAP DH*; 5′-ATGGGGAAGGTGAAGGTCG-3′ and 5′-TAAAAGCAGCCCTG GTGACC-3′ (70 bp product) as an internal control. A fluorescence signal was collected at the end of each cycle. After the reactions were terminated, the signal at each temperature from 60 to 95 °C was also collected for the dissociation curve analysis. All reactions were performed in triplicate to confirm reproducibility, and the amount of target mRNA in each sample was normalized with that of mean *GAPDH*.

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