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ISOLATION AND EVALUATION OF HEDGEHOG INHIBITORS FROM CHRISTMAS GRASS (*THEMEDA ARGUENS*)

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Abstract – The hedgehog (Hh) signaling pathway is an important regulator of embryonic development, tissue patterning, cellular proliferation and differentiation. However, aberrant activation of the pathway is associated with tumorigenesis. In this study, the Hh signal inhibitory activity of plant extracts was measured with a cell-based assay system that targets GLI1-mediated transcription. Activity guided analysis of MeOH extracts of christmas grass (*Themeda arguens*) led to the isolation of four inhibitory compounds: aciculatin (**1**), 7-de-*O*-methylaciculatin (**2**), 8-*C*-β-D-boivinopyranosylapigenin (**3**) and aciculatinone (**4**). Compound **1** strongly inhibited Hh/GLI1-mediated transcriptional activity with an IC_{50} value of 1.8 μM, and disrupted the formation of the GLI1-DNA complex by EMSA. **1** and **3** were cytotoxic for human prostate (DU-145) and breast (MCF-7) cancer cells. These compounds may serve as new tools for the molecular dissection of Hh pathway activation.

Dedicated to Professor Masakatsu Shibasaki on his 70th birthday

INTRODUCTION

The hedgehog (Hh) signaling pathway regulates proliferation, differentiation and tissue patterning during embryonic development, and is involved in the maintenance of homeostasis in numerous tissues and organs.[1] Hyper-activation caused by overexpression of component proteins or mutations in genes encoding key members of the pathway results in many human developmental disorders, particularly cancers, including basal cell carcinoma (BCC), medulloblastoma, rhabdomyosarcoma, pancreatic cancer

and prostate cancer.[2-4] There are three Hh genes in mammals, Sonic hedgehog, Desert hedgehog and Indian hedgehog. Sonic hedgehog is the most broadly expressed and well studied of the three ligands.

Hh ligands are released into the extracellular milieu after sequential post-translational modifications, including autocleavage of the C-terminal domain, C-terminal cholesterylation, and palmitoylation of the N-terminal domain by Hedgehog acyltransferase (Hhat). In the absence of Hh ligand, the 12-transmembrane protein patched 1 (PTCH1) inhibits the cell-surface localization of the seven-trans membrane protein smoothened (SMO) on the primary cilium (Figure 1). At the base of the cilium, the GLI transcription factors are phosphorylated by casein kinase 1 (CK1), glycogen synthase kinase 3β (GS K3β) and cAMP-dependent protein kinase A (PKA). Phosphorylated GLIs are proteolytically cleaved to generate the repressive GLI factors, GLI2R and GLI3R, which subsequently translocate to the nucleus to suppress the transcription of Hh target genes. Upon binding of Hh ligand to PTCH1, PTCH1 releases its inhibition of SMO. SMO then moves to the primary cilium and initiates the activation of GLI proteins, which translocate to the nucleus and drive the transcription of important proliferation genes such as *ptch1*,

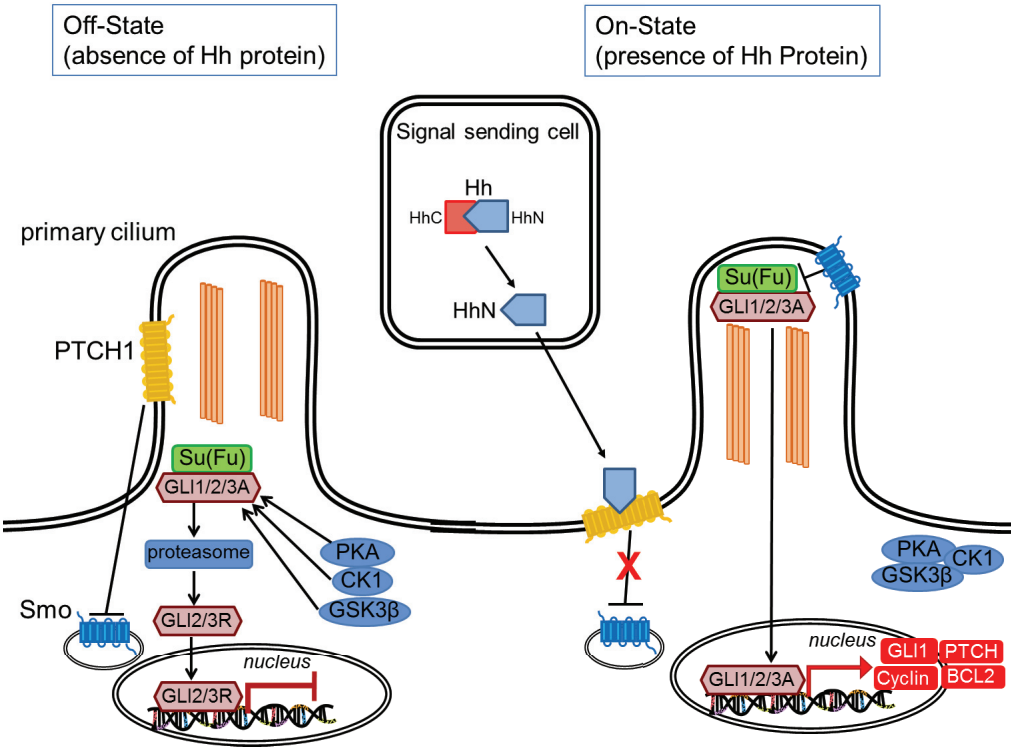

Figure 1. The Hh signaling pathway. In the absence of Hh protein (off-state), SMO is prevented from entering the primary cilium by PTCH1. GLIs are phosphorylated by kinases, PKA, CK1, and GSK3β to form GLIR and enter the nucleus to suppress the transcription of target genes. Hh protein is released into the extracellular milieu after sequential post-translational modifications. In the presence of Hh protein, the suppression of SMO by PTCH1 is diminished. SMO is activated and translocates to the primary cilium. Su(Fu) is inhibited by SMO and GLIA enters the nucleus and induces the transcription of target genes. PTCH1; patched1, SMO; smoothened, Su(Fu); suppressor of fused, PKA; protein kinase A, CK1α; casein kinase 1α, GSK3β; glycogen synthase kinase-3β, GLIR; inactive form of GLI, GLIA; active form of GLI, HhN; N-terminal domain of Hh protein, HhC; C-terminal domain of Hh protein.

gli1, *cyclin D*, *cyclin E*, *N-Myc*, *VEGF* and *Bcl2*. Since the growth of several cancers depends on activation of Hh signaling,[5] inhibition of the pathway is an attractive approach to cancer treatment. Clinical trials with several SMO and GLI1 inhibitors against BCC and other tumors have been performed, and others are underway.[6,7]

Several Hh inhibitors have been described. The first of these was the teratogenic alkaloid, cyclopamine, which is SMO inhibitor isolated from *Veratrum californicum* (Liliaceae).[8-10] Other SMO antagonists were subsequently developed, including the SANTs,[11] CUR61414,[12] and vismodegib, which was the first Hh inhibitor approved by the USFDA for the treatment of adult BCCs.[13] In addition, several types of Hh inhibitors that do not target SMO have been described, such as RU-SKI 43 (a Hhat inhibitor of sonic hedgehog N-terminal fragment post-translational modification),[14] Robotnikinin, (another sonic hedgehog N-terminal fragment inhibitor),[15] and HPI (an inhibitor of AAA+ ATPase motor cytoplasmic dynein).[16] The small molecule inhibitor, GANT61, has been reported to induce modification of the GLI1 protein and inhibit GLI1 mediated transcriptional activation.[17] Arsenic trioxide, which is approved for the treatment of acute promyelocytic leukaemia, reportedly inhibits Hh signaling by reducing GLI2 stability.[18]

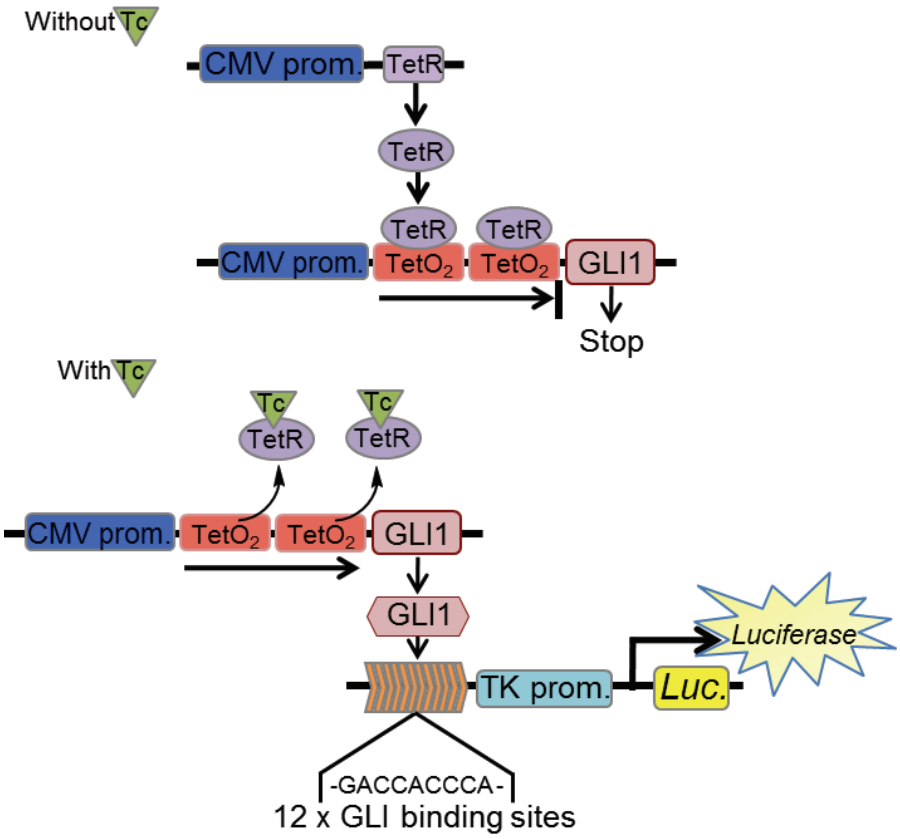

Figure 2. Schematic depiction of the assay system. Without tetracycline (Tc), the Tet repressor (TetR) regulates expression of exogenous GLI1. With Tc, Tc inactivates the TetR to facilitate GLI1 expression. GLI1 binds to the GLI binding site to initiate luciferase (Luc.) transcription, which is measured as a read-out of GLI1 transcriptional activity. TetO; tetracycline operator; CMV prom; cytomegalovirus promoter; TK prom; thymidine kinase promoter.

Previously, we developed a cell-based assay system for assessing Hh signaling pathway activity,[19] based on the tetracycline-regulated (T-Rex) modulation of GLI1 activation (Figure 2). We isolated several naturally occurring Hh inhibitors from various species of plants, such as physalin B, physalin F,[19] colubrinic acid,[20] taepeenin D,[21] caldenolides,[22] flavonoid glycoside,[23] vitetrifolin D,[24] and physalin H,[25] and withaferin A[26] by bioactivity-guided isolation using this assay. Continuing with this approach, we assayed whole plant extracts from *Themeda arguens*, a poaceous plant, also known as christmas grass or kangaroo grass, which is distributed throughout tropical Asia, Australia and South America. While *T. arguens* extracts are used for the treatment of rheumatism,[27] to the best of our knowledge, there has been no research addressing the bioactive components of this plant.

In this screening study, we utilized our cell-based luciferase assay method to evaluate the capacity of *Themeda arguens* extracts to inhibit Hh signaling. Using this activity-guided approach, four inhibitory compounds (**1**-**4**) were isolated.

RESULTS AND DISCUSSION

In our screening study of a plant extract library, we identified a MeOH extract of *T. arguens* that inhibited GLI1-mediated transcription using our assay system.[19] A 50 μg/mL MeOH extract reduced luciferase activity to 50%, without affecting the viability of the reporter assay cells, suggesting that the decrease in luciferase activity was not due to extract cytotoxicity. The composition of the *T. arguens* MeOH extract was investigated using chromatographic techniques (Diaion HP-20, silica gel and ODS), resulting in the isolation of aciculatin (**1**),[28] 7-de-*O*-methylaciculatin (**2**),[29] 8-*C*-β-D-boivinopyranosylapigenin (**3**)[29] and aciculatinone (**4**)[29] (Figure 3). Compounds **1**-**4** were identified by comparing their spectroscopic data to published values. The compounds inhibited GLI1-mediated transcription in a dose-dependent manner (Figure 4, Table 1). Compound **1** exhibited strong inhibitory activity with an IC_{50} value of 1.8 μM. Comparisons of the inhibition of GLI1-mediated transcription of **1** with **2** and **3** highlighted the importance of the methoxy group at position 7 for the activity. Furthermore, the lower activity of **4** compared with **1** indicated that the hydroxy group at the 3" position of the sugar moiety was potentially important for Hh signaling inhibition. Hh signling is activated in synovial tissue in active rheumatoid arthritis, and associated with the proliferation of fibroblast-like synoviocytes in rheumatoid arthritis, which induces joint destruction.[30] *T. arguens* was used for treatment of rheumatism. This therapeutic effect may be due to these compounds. In addition, these compounds were reported to show cytotoxicity against several cancer cell lines.[29]

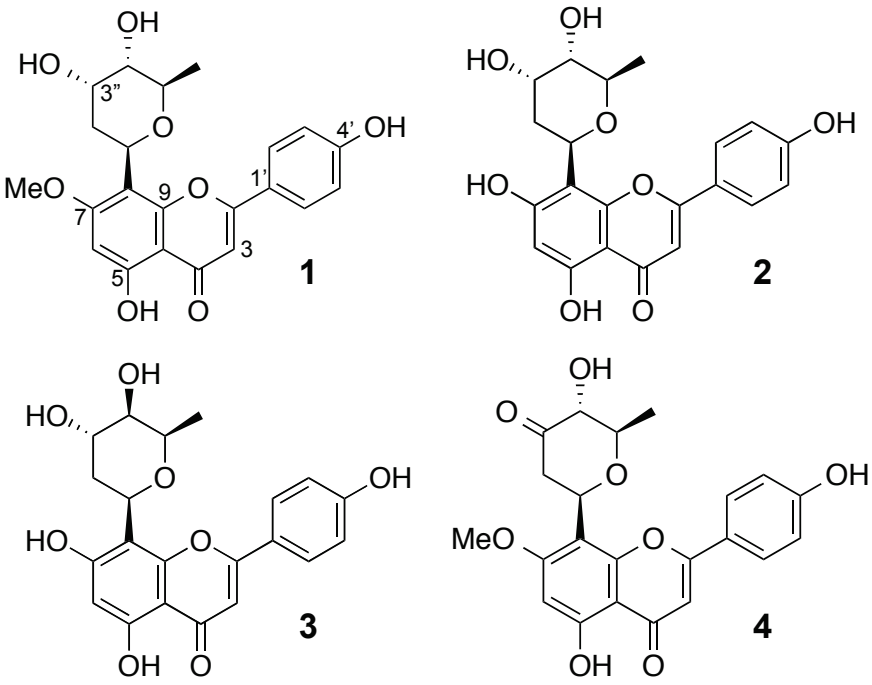

Figure 3. Compounds isolated from whole plant extracts of *T. arguens*

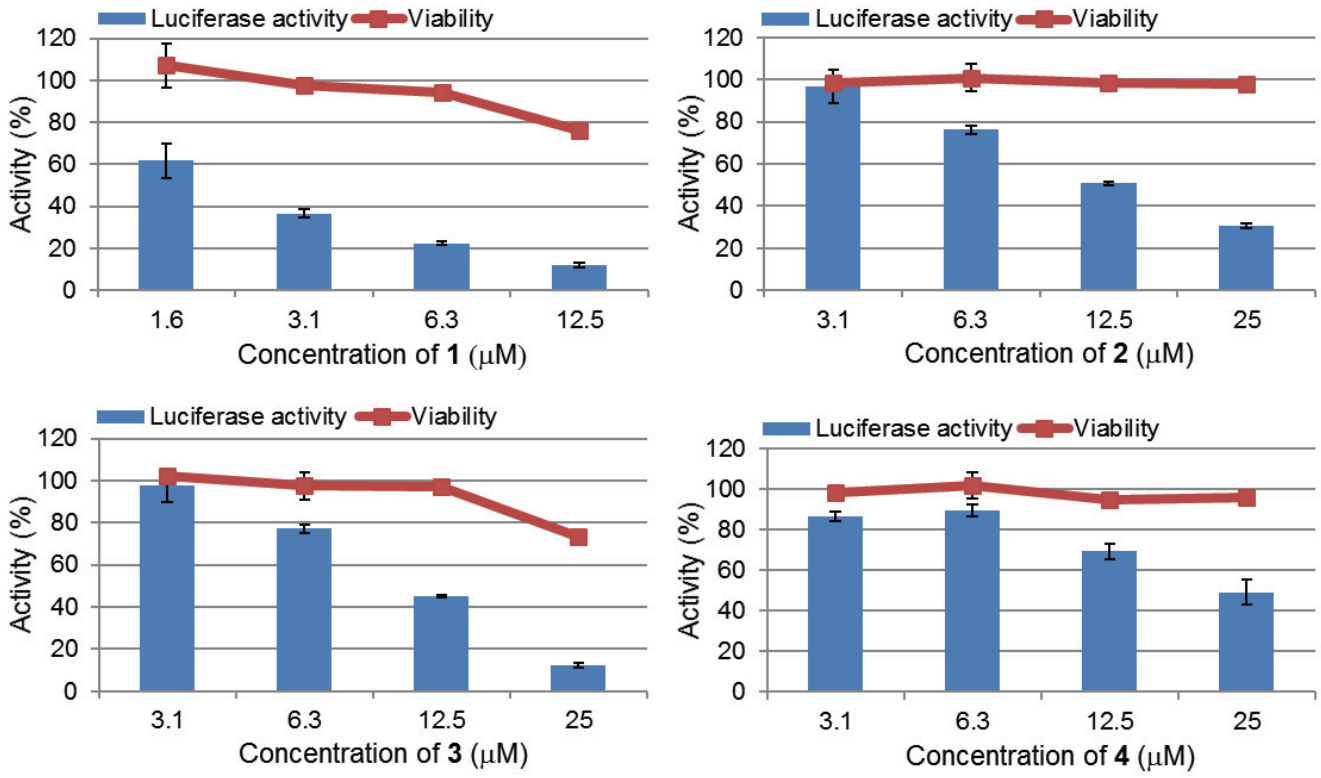

Figure 4. GLI1 transcriptional inhibitory activity and cell viability after treatment with compounds **1**-**4**. The assays were performed in 0.05% DMSO (n = 3). Error bars represent S.D.

Table 1. GLI1 transcriptional inhibitory activity of compounds **1**-**4**

Compound	GLI1 transcriptional inhibition (IC_{50} μM)
1	1.8
2	13.0
3	10.6
4	24.3

Next, we investigated the cytotoxicity of the isolated compounds against a panel of cancer cell lines that were reported to have aberrant activated Hh signaling, including human pancreatic cancer cells (PANC-1), prostate cancer cells (DU-145), and breast cancer cells (MCF-7) (Table 1, Figure 5). We also determined the cytotoxicity of the isolated compounds using the murine mesenchymal stem cell line C3H10T1/2, a normal cell line that is responsive to Hh signaling. **1** and **3** showed cytotoxicity against DU-145 cells and MCF-7 cells, while they did not show cytotoxicity against C3H10T1/2 cells, which was consistent with inhibition of aberrant Hh signaling pathway.

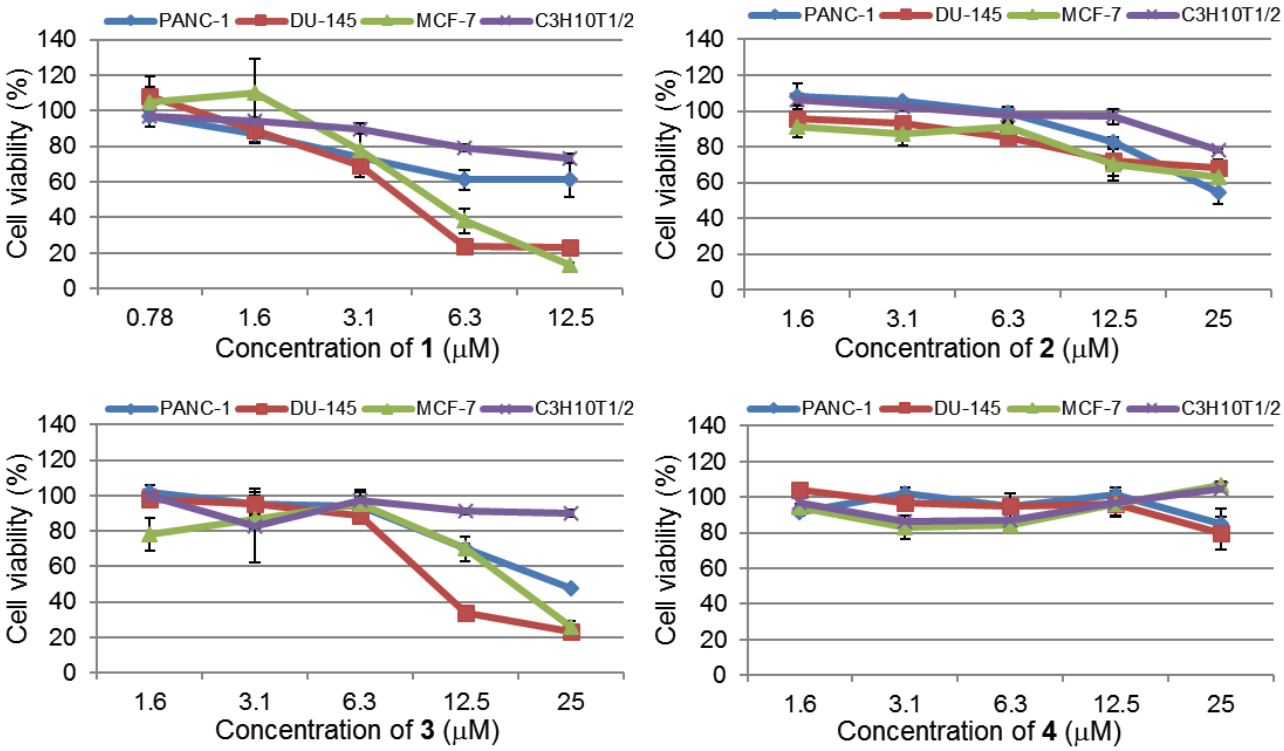

Figure 5. Cytotoxic effects of **1**-**4** on PANC-1 cells, DU-145 cells, MCF-7 cells, and C3H10T1/2 cells. The assays were performed in 0.05% DMSO (n = 3). Error bars represent S.D.

Table 2. IC_{50} of compounds **1**-**4** for PANC-1 cells, DU-145 cells, MCF-7 cells, and C3H10T1/2 cells

Compound	Cytotoxicity (IC_{50} μM)			
	PANC-1	DU-145	MCF-7	C3H10T1/2
1	>12.5	4.6	5.4	>12.5
2	>25	>25	>25	>25
3	23.8	7.8	16.5	>25
4	>25	>25	>25	>25

Western blot analyses were used to address the the effects of **1** on the expression levels of PTCH1 and BCL2 proteins, both of which are expressed in the HaCaT and DU-145 cell lines (Figure 6). At a concentration of 3 μM, densitometry analysis revealed that **1** decreased the level of BCL2 without affecting the over expression of GLI1 protein in HaCaT cells. Moreover, **1** dose dependently inhibited the expression of PTCH1 and BCL-2 in DU-145 cells.

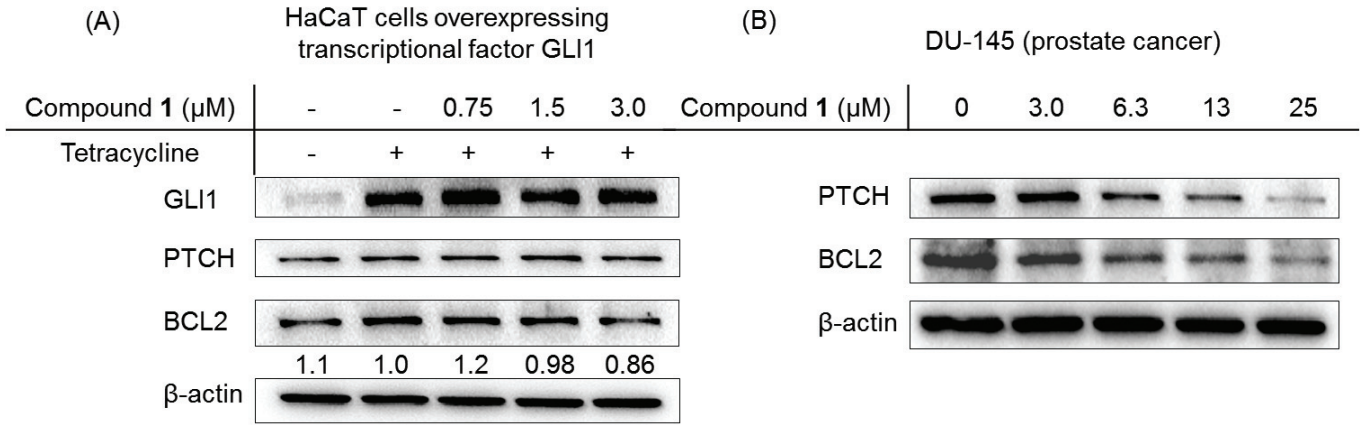

Figure 6. Analysis of PTCH1 and BCL2 protein expression in HaCaT and DU-145 cells treated with compound **1**. (A) Western blot analysis of GLI1, PTCH1, and BCL2 protein levels in HaCaT assay cells after treatment with **1**. (B) Western blot analysis of PTCH1, and BCL2 protein levels in DU-145 cells after treatment with **1**.

Previously, we developed an electrophoretic mobility shift assay (EMSA) to evaluate the effects of compounds on GLI1-DNA complex formation.[24,25] Horseradish peroxidase (HRP)-conjugated streptavidin was applied for detection of biotin-labelled DNA containing the GLI1 binding site (5'-biotin-AGCTACCTGGGTGGTCTCTTCGA-3, the underlined nucleotides indicate the GLI1 consensus sequence). Using this assay system, we evaluated the effect of aciculatin (**1**) and 8-*C*-β-D-digitoxopyranosylapigenin (**2**), which was a weak inhibitor, on formation of the GLI1-DNA complex containing the GLI1 binding site. As shown in Figure 7, **1** inhibited the formation of the GLI1-DNA complex at 200 μM, whereas the weak inhibitor **2** did not significantly affect complex

formation. The EMSA result suggested that the Hh inhibitory activity of **1** involves the inhibition of GLI1-DNA complex formation. This is consistent with the previously reported DNA-binding activity of aciculatin.[27] Conversely, the inability of **2** to affect GLI1-DNA complex formation may explain its relatively weak inhibition of GLI1-mediated transcription.

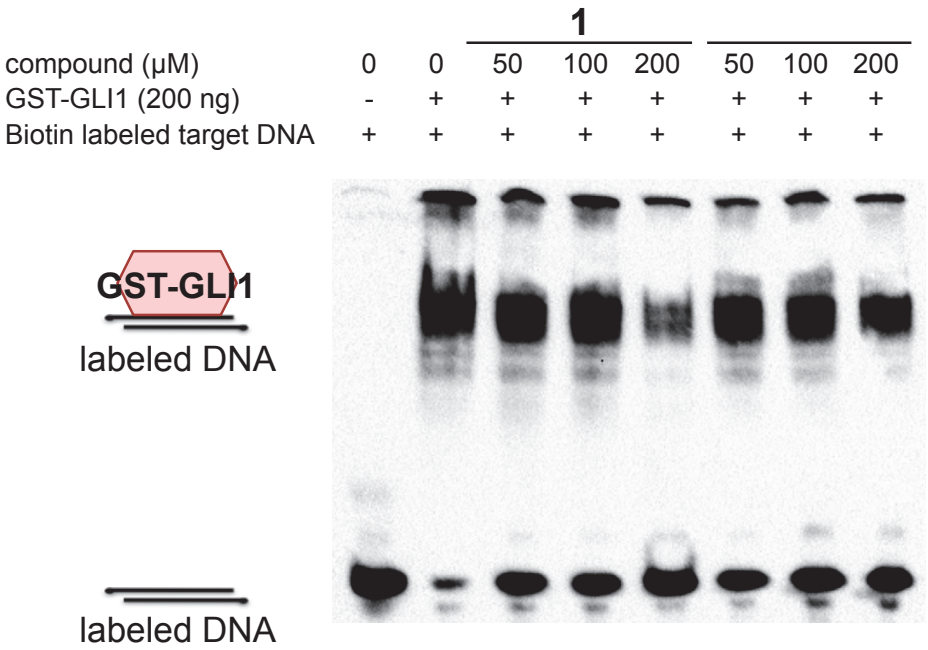

Figure 7. Analysis of the effect of compounds **1** and **2** on GLI1-DNA complex formation by EMSA. A recombinant GST-GLI1 protein (aa 171-515), and DNA containing the GLI1 binding site; were used in the binding reaction. Reproducibility was determined in three individual experiments.

In conclusion, we identified four GLI-mediated transcriptional inhibitors from whole plant extracts of *T. arguens*. Compounds **1** and **3** showed cytotoxicity toward Hh signaling-positive cancer cell lines (DU-145, MCF-7). **1** suppressed the expression of Hh signaling pathway target proteins, Hh ligand receptor PTCH1, and anti-apoptosis protein BCL2. These natural compounds represent significant new additions to the growing global repository of Hh pathway inhibitors. Ultimately, some of these compounds may provide useful laboratory tools for dissecting the molecular details of Hh pathway activation.

EXPERIMENTAL

General Experimental Procedures

NMR spectra were recorded on a JEOL ECA-600 and an ECP-600 with deuterated solvent ($CDCl_3$, CD_3OD and acetone-d_6). DNA concentrations were measured using a Nano Drop 2000 (Thermo Fisher Scientific, USA), and protein concentrations were measured using an UVmini 1240 UV-vis Spectrophotometer (SHIMAZU, Japan). High-performance liquid chromatography (HPLC) was performed using an LC-2000 Plus series (JASCO, Tokyo, Japan). The GST-GLI1 protein was produced

as previously described.[19,20]

Plant Material

Themeda arguens (Poaceae) specimens were collected in Thailand (2008). A voucher specimen (KKP259) was deposited at the Department of Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, Chiba University, Japan.

Extraction and Isolation

Whole *Themeda arguens* plants (183.9 g) were extracted with MeOH. The MeOH extract (15.4 g) was subjected to Diaion HP-20 column chromatography (ϕ 10×230 mm) and eluted with a gradient mixture of H_2O:MeOH (1:1-1:0) and MeOH:acetone (1:1-0:1) to yield fractions 1A to 1F. Fraction 1D (2.2 g), which was eluted with MeOH, showed Hh inhibitory activity and was chromatographed over a silica gel column ϕ 60×240 mm) using $CHCl_3$:MeOH mixtures (1:0-0:1, 0:1 + 0.1 % TFA) to give fractions 2A-2N. Fraction 2E (44.0 mg) was chromatographed on a ODS column (ϕ 12×220 mm) using H_2O:MeOH mixtures of decreasing polarity to yield fractions 3A-3I. Fraction 3E (11.2 mg) was purified with Develosil ODS-MG-5 (ϕ 10×250 mm, eluent MeOH:H_2O (6:4), flow late 3.0 mL/min, UV 254 nm) to yield **1** (4.8 mg, t_R 45 min) and **2** (2.3 mg, t_R 49 min). Fraction 2F (31.0 mg) was subjected to ODS column chromatography (ϕ 12×220 mm) using MeOH:H_2O mixtures of decreasing polarity to give fractions 4A-4I. Fraction 4G (8.7 mg) was purified with Develosil ODS-MG-5 (ϕ 10×250 mm, eluent MeOH:H_2O (53:47), flow late 3.0 mL/min, UV 254 nm) to yield **2** (2.4 mg, t_R 64 min) and **3** (1.3 mg, t_R 69 min). Fraction 1E (2.5 g) was subjected to silica gel column chromatography (ϕ 25×240 mm) using $CHCl_3$:MeOH mixtures of increasing polarity to give fractions 5A-5M. Fraction 5C (159.9 mg) was subjected to ODS column (ϕ 18×300 mm) with H_2O:MeOH (1:1-0:1, 0:1 + 0.1 % TFA) to yield fractions 6A-6M. Fraction 6E (10.2 mg), which was eluted with MeOH:H_2O (1:1), was further purified with YMC-Pack ODS-AM (ϕ 10×250 mm, eluent MeOH:H_2O (6:4), flow late 3 mL/min, UV 254 nm) and Sephadex LH-20 column chromatography (ϕ 12×340 mm) with MeOH to produce **4** (1.1 mg). Fraction 3E (266.0 mg) was subjected to ODS column chromatography (ϕ 24×230 mm) using H_2O:MeOH (7:3-0:1) to give fractions 7A-7I. Fraction 7F (45.3 mg), which was eluted with MeOH:H_2O (6:4), was subjected to Sephadex LH-20 column chromatography (ϕ 15×430 mm) using $CHCl_3$:MeOH (1:1) to give fractions 8A-8D. Fraction 8B (23.7 mg) was purified with Cosmosil 5CN-MS (ϕ 10×250 mm, eluent MeOH:H_2O (45:55), flow late 3 mL/min, UV 254 nm, nacalai tesque, Japan) to yield **1** (16.3 mg, t_R 12 min). Fraction 8C (20.2 mg) was purified with YMC-Pack ODS-AM (ϕ 10×250 mm, eluent MeOH:H_2O (6:4), flow late 3 mL/min, UV 254 nm, YMC, Japan) to yield **2** (12.7 mg, t_R 66 min).

Cell culture

Assay cells were cultured in DMEM high glucose (Wako, Japan) with 5% fatal bovine serum (FBS, Bio West) and 200 unit/mL penicillin and 200 μg/mL streptomycin (Gibco, USA). PANC-1 cells, DU-145 cells were cultured in RPMI (Wako) containing 10% FBS. MCF-7 cells and C3H10T1/2 cells were cultured in DMEM-high glucose medium containing 10% FBS. PANC-1 cells, MCF-7 cells, and C3H10T1/2 cells were obtained from RIKEN BRC. DU-145 cells were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University.

GLI1-Mediated Transcriptional Activity Assay

Assay cells (HaCaT cells-expressing exogenous GLI1 under control of Tc[19] were cultured in a 96-well white plate (Thermo Fisher Scientific) in 100 μL of DMEM-high glucose containing 5% fatal bovine serum, 200 unit/mL penicillin and 200 μg/mL streptomycin at 2×10^4 cells/well at 37 °C for 12 h. After incubation, 1 μg/mL of tetracycline was added to each well to induce exogenous GLI1 protein expression, and cells were incubated at 37 °C for 12 h. The medium was replaced with tetracycline-free medium containing individual samples. 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. At the same time, the viability of the sample-treated cells was measured using a Fluorometric Microculture Cytotoxicity Assay (FMCA).[31]

Assay cells were cultured in a 96-well black plate (Thermo Fisher Scientific) at 2×10^4 cells/well at 37 °C for 24 h. Samples were added at the same time as the luciferase assay (24 h after seeding), and the cells were incubated at 37 °C for 12 h. Cell viability was determined with FMCA using a fluorescence plate reader (Thermo). Cell viability was calculated as the ratio of viable sample-treated cells to non-treated cells. Detailed procedures have been described previously.[25]

Cytotoxicity test

Cancer cells (PANC-1, DU-145, and MCF-7) or normal cells (C3H10T1/2) were added to 96-well black plates in 100 μL of RPMI (Wako) containing 10% FBS (for PANC-1 cells and DU-145 cells) or DMEM-high glucose medium containing 10% FBS (for MCF-7 and C3H10T1/2) at 1×10^4 cells per well and incubated at 37 °C for 24 h. The medium was then replaced with fresh medium containing different concentrations of individual test compounds. After incubation for 24 h, the medium was removed and cell proliferation was determined by FMCA using a fluorescence plate reader (Thermo).

Western Blotting Analysis

Assay cells (HaCaT cells-expressing exogenous GLI1) or DU-145 cells were added to a 6 cm dish in 5 mL of DMEM-high glucose containing 5% FBS, 200 unit/mL penicillin and 200 μg/mL streptomycin (for assay cells) or RPMI medium containing 10% FBS (for DU-145 cells) at 1×10^6 cells per dish and incubated for 12 h at 37 °C. To activate expression of exogenous GLI1 protein in assay cells, 1 μg/mL of tetracycline was added into each dish, followed by a further 12 h incubation. The medium was then removed and fresh medium containing individual compounds was added. After a 12 h incubation, cells were washed twice with 500 μL PBS and collected by scraping the dishes. Protein lysates were prepared in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 10 mM EDTA, 1 mM sodium orthovanadate, and 0.1 mM NaF) containing a 1% proteasome inhibitor cocktail (Nacalai Tesque, Tokyo, Japan). Protein lysates were subjected to 5% or 12.5% SDS-PAGE electrophoresis and subsequently transferred to PVDF membranes (Bio-Rad). Blots were blocked with TBST (10 mM Tris-HCl pH 7.4, 100 mM NaCl and 0.1% Tween 20) containing 5% skimmed milk for 1 h and incubated at 4 °C for 12 h with primary antibodies (anti-GLI1 ((C-18): sc-6152), 1:200, Santa Cruz Biotechnology, USA; anti-PTCH (ab53715), 1:500, Abcam, Japan; anti-BCL-2 (B9804), 1:2000, Sigma-Aldrich, USA; anti-β-actin (A2228), 1:4000, Sigma-Aldrich). The membranes were then washed with TBST and incubated at room temperature for 1 h with secondary antibodies, either anti-goat IgG (A-5420), 1:10000, Sigma-Aldrich, anti-rabbit IgG (111-035-144), 1:4000, Jackson Immuno Research, USA, or anti-mouse IgG (NA931), 1:4000, GE Healthcare/Amersham Biosciences, USA). After washing with TBST, immunocomplexed bands were detected using an ECL Advance Western (GE Healthcare Biosciences) or an Immobilon Western (Millipore) detection system.

Electrophoretic mobility shift assay

The assays were performed with a Light Shift Chemiluminescent EMSA Kit (Thermo). The binding reaction was undertaken as follows: Recombinant GST-GLI1 protein (aa 171-515, 200 ng), biotin-labelled GLI1-binding site (20 fmol), and the test compound in DMSO (1% final) were combined in 2 μL of 10 × binding buffer, 5 mM $MgCl_2$, and 4% glycerol on ice (total volume 20 μL). After incubation for 20 min, protein-DNA complexes, free DNA, and free proteins were separated on a 6% native polyacrylamide gel containing 45 mM tris-borate and 1 mM EDTA. After transferring the DNA onto a Biodyne B nylon membrane (0.45 μm, Pall Gelman Laboratory, USA) and crosslinking the DNA with a UV transilluminator (312 nm, 15 min), biotin-labelled DNA was detected with HRP-conjugated streptavidin, following the manufacturers' protocols. Detailed procedures have been described previously.[24]

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