Terpenoids and a Flavonoid Glycoside from *Acacia pennata* Leaves as Hedgehog/GLI-Mediated Transcriptional Inhibitors

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Overexpression of glioma-associated oncogene 1 (GLI1), which has been characterized as a terminal effector and a target gene of the Hedgehog (Hh) signaling pathway, is associated with the development of cancer. A cellular screen was applied utilizing of a GLI-dependent luciferase reporter in human keratinocyte cells (HaCaT) and identified two terpenoids (**1** and **2**) and a flavonoid glycoside (**5**) from *Acacia pennata* as Hh/GLI inhibitors. Compounds **1**, **2**, and **5** exhibited selective cytotoxicity against human pancreatic (PANC1) and prostate (DU145) cancer cells with no toxic effect on normal cells. This result was consistent with a dose-dependent reduction of the protein levels of antiapoptotic BCL-2 and the tumor suppressor patched 1 protein (PTCH). Additionally, treatment of **1** downregulated mRNA expression of Ptch in PANC1, suggesting that the compound has an inhibitory effect on the transcription of Hh/GLI.

Hedgehog (Hh) signaling regulates numerous events in embryonic development and adult tissue maintenance. Defects in Hh signaling lead to birth defects in mice and humans and is aberrantly associated with the development of various cancers.[1] Hh signaling transduction begins with the binding of Hh protein ligand to its membrane receptor PTCH. This binding abolishes the inhibitory effect of PTCH on Smoothened (Smo), allowing Smo to activate the GLI family of transcription factors; therefore aberrant Hh signaling leads to tumor formation and progression,[2] including cancers such as of the pancreas[3] and prostate.[4]

Several synthetic and naturally occurring Hh signaling antagonists, such as cyclopamine,[5,6] SANTs,[7] and CUR61414,[8] have been discovered previously. Inhibitors that act downstream of Smo (GANTs[9]) that inhibit alcohol dehydrogenase 7 (JK184[10]), as well as a small molecule that binds sonic hedgehog (robotnikinin[11]), have also been reported. Despite these initial leads, there remains an urgent need to find more potent Hh signaling inhibitors.

As part of an ongoing program seeking Hh/GLI-mediated transcriptional inhibitors, a previously constructed cell screen of expressed exogenous GLI1 in HaCaT under tetracycline control (T-REx system) was used to screen natural product libraries.[12,13] To identify the cell viability of compounds, a fluorometric microculture cytotoxicity (FMCA) method[14] was used. Herein, we report Hh/GLI-mediated transcriptional inhibitors isolated from *Acacia pennata* Willd. (Leguminosae).

A methanol extract of *A. pennata* was partitioned with hexane, EtOAc, *n*-BuOH, and H_2O. Among these, hexane and EtOAc extracts demonstrated potent Hh/GLI signaling inhibition at 50 μg/mL. The percentages of their luciferase activities were 13% and 10%, respectively, with cell viability more than 49%. Both active extracts were then subjected to silica gel column chromatography, followed by Sephadex LH-20 and ODS chromatography, to yield taepeenin D (**1**),[15] (+)-drim-8-ene (**2**),[16] labdanolic acid (**3**),[17] 8,15-labdanediol (**4**),[18] and quercetin 3-*O*-β-D-glucopyranosyl-4-*O*-β-D-glucopyranoside (**5**)[19] (Figure 1). Compounds **1**−**5** were identified by comparing their spectroscopic data to literature values. Compounds **1**, **2**, and **5** inhibited Hh/GLI-mediated transcriptional activity with IC_{50} values of 1.6, 13.5, and 10.5 μM, respectively, whereas compounds **3** and **4** were inactive (Figure 2, Table 1).

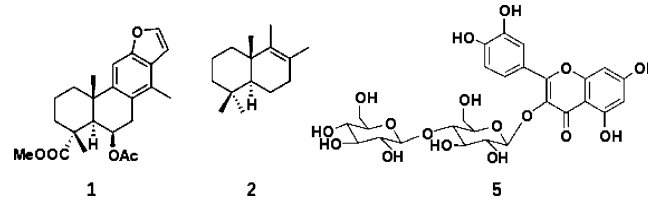

Figure 1. Hedgehog/GLI-mediated inhibitors from *Acacia pennata* leaves: taepeenin D (**1**), (+)-drim-8-ene (**2**), and quercetin 3-*O*-β-D-glucopyranosyl-4-*O*-β-D-glucopyranoside (**5**).

Table 1. IC_{50} Values of GLI-Mediated Transcriptional Inhibition and Cytotoxicity against Cancer Cells (PANC1, DU145) and C3H10T1/2 Cells

compound	GLI1 transcriptional inhibition (IC_{50}; μM)	cytotoxicity (IC_{50}; μM)		
		PANC1	DU145	C3H10T1/2
1	1.6	3.2	3.4	>100
2	13.5	15.1	23.2	>100
5	10.5	26.6	30.0	>100

As for cytotoxicity assays, a panel of cells was included with increased Hh signaling levels (PANC1 and DU145) and without reliance on the Hh ligand for survival (C3H10T1/2). The results revealed that compounds **1**, **2**, and **5** were cytotoxic against PANC1 cells (IC_{50} values of 3.2, 15.1, and 26.6 μM, respectively) and DU145 cells (IC_{50} values of 3.4, 23.2, and 30.0 μM, respectively) but did not affect normal cell lines (Figure 3, Table 1).

GLI1 transcriptional factors are known to regulate expression of multiple targets including PTCH[20] and BCL-2.[21] Both proteins were overexpressed in HaCaT-GLI1-Luc cells and PANC1. Although compounds **1**, **2**, and **5** differently interfered with PTCH and BCL-2 proteins, western blot analysis confirmed that each compound reduced the expression of both proteins in a concentration-dependent fashion (Figure 4). It is also important to note that the GLI1 protein level in HaCaT cells decreased for compound **1** but not for compounds **2** and **5**. In this assay, tetracycline, which introduces exogenous GLI1 overexpression, was removed when compounds were added to HaCaT-GLI1-Luc cells. Therefore, compound **1** might accelerate GLI1 protein degradation in HaCaT-GLI1-Luc cells. These results thus indicated that different structures had different target molecules.

Furthermore, the inhibition of Ptch mRNA expression by taepeenin D (**1**) in PANC1 was examined using real-time PCR

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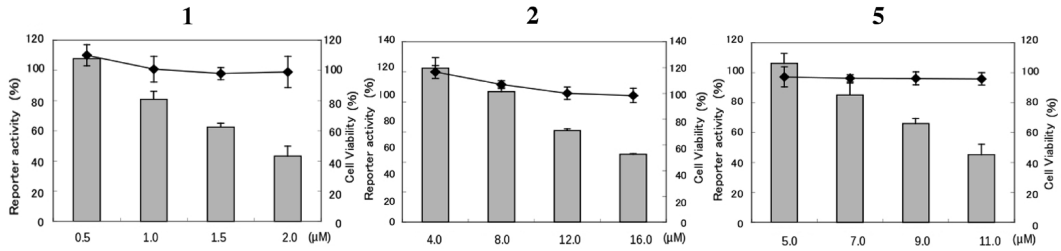

Figure 2. Inhibition of GLI1-mediated transcriptional activity (solid columns) and cell viability (solid curves) of compounds **1**, **2**, and **5**. HaCaT-GLI1-Luc cells were seeded onto a 96-well plate (2×10^5 cells per well) and then treated with compounds after 12 h tetracycline addition. Cell viability and luciferase activity were determined at the same time. The assays were performed at 0.05% DMSO ($n = 3$). Error bars represent SD.

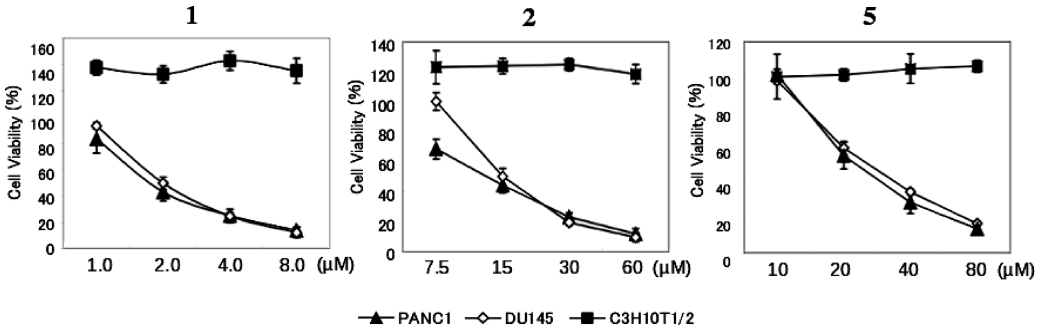

Figure 3. Cytotoxicity of compounds **1**, **2**, and **5** against PANC1, DU145, and C3H10T1/2 cells. The assays were performed with 0.05% DMSO ($n = 3$). Error bars represent SD.

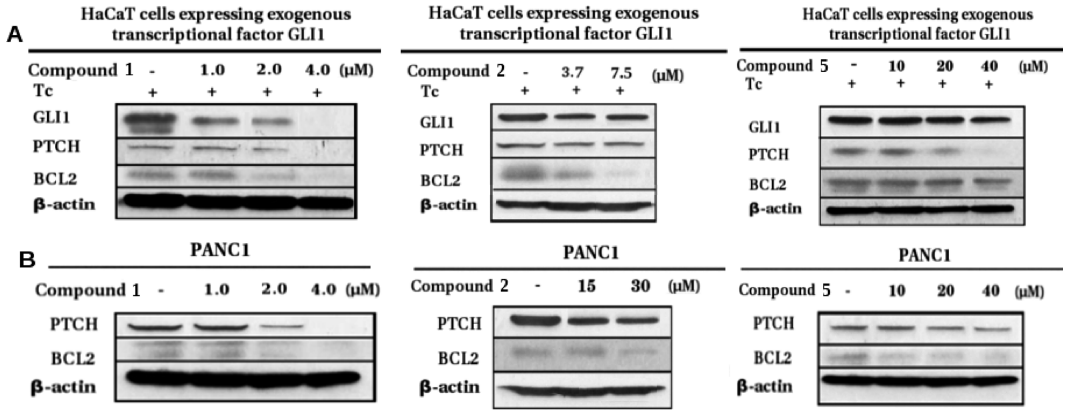

Figure 4. Inhibition of GLI-related protein (PTCH and BCL2) levels by compounds **1**, **2**, and **5**. (A) Treatment of **1**, **2**, and **5** on GLI1-overexpressing HaCaT cells. (B) Treatment of **1**, **2**, and **5** on PANC1 cells.

analysis. Cyclopamine, a steroidal alkaloid that inhibits the Hh response,[6,22] was included as a positive control. The antagonism of Smo was reported previously as a target of cyclopamine action.[23,24] As depicted in Figure 5, treatment with **1** at 2 and 4 μM reduced the mRNA expression of Ptch compared to a negative control. A similar trend of inhibition was apparent on the mRNA expression of cyclopamine-treated cancer cells. A report by Chatel et al. highlighted the fact that treatment of cyclopamine (10 μM) modulated Ptch expression in PANC1 due to the inhibition of the Gli1 mRNA level,[25] thereby suggesting that taepeenin D (**1**) is also an effective inhibitor of the Hh signaling pathway.

In conclusion, three constituents of *A. pennata* leaves were found to inhibit Hh signaling. This inhibition was caused by downregulated Ptch mRNA expression along with decreased protein level of PTCH and BCL-2 in HaCaT cells expressing exogenous GLI1 and PANC1 cells. Therefore, compounds **1**, **2**, and **5** may be valuable as a probe of diseases related to Hh/GLI-dependent cancer.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a JEOL A-400 and an ECP-600 with deuterated solvent ($CDCl_3$ and CD_3OD).

Plant Material. The leaves of *A. pennata* were collected in Thailand and were sent to Japan in February 2008. The plant was identified by one of the authors (T.K.), and a voucher speciment (7-300) has been deposited in both Khon Kaen University (Thailand) and the Graduate School of Pharmaceutical Sciences, Chiba University (Japan).

Extraction and Isolation. Leaves (10.0 g) of *A. pennata* were extracted with MeOH. The MeOH extract was partitioned with hexane (200 mL × 3), EtOAc (200 mL × 3), and *n*-BuOH (200 mL × 3). The EtOAc-soluble fraction (1.9 g) was subjected to silica gel column chromatography (40 × 260 mm) and eluted successively with a gradient mixture of hexane−EtOAc−MeOH (10:2:1 to 2:2:1) to yield seven fractions (1A to 1G). Fraction 1D (390.3 mg) was chromatographed over a 60 N silica gel column (12 × 245 mm) using hexane−EtOAc−MeOH mixtures of increasing polarity to give fractions 2A−2J. Active fraction 2E (131.2 mg) was further separated by PSQ-100B silica gel column chromatography (30.3 mg), followed by passage over Sephadex LH-

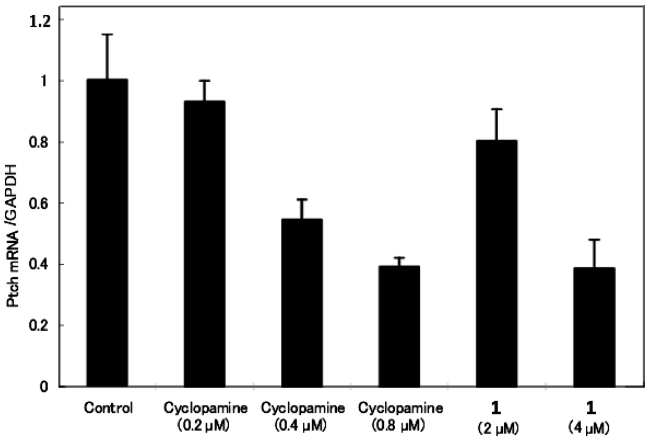

Figure 5. Inhibition of GLI1-mediated mRNA expression of Ptch by compound **1** in PANC1 cells. GAPDH was used as an internal control. The assays were performed at 0.05% DMSO ($n = 3$). Error bars represent SD.

20 (10 × 150 mm), using methanol 100%, to afford compounds **1** (13.8 mg), **2** (3.0 mg), and **3** (3.7 mg). Fraction 4D (60.7 mg), separated from the hexane extract (0.98 g), was chromatographed over 60 N silica gel (20 × 450 mm), using hexane−$CHCl_3$−EtOAc (from 16:1:1 to 0:2:1), to yield compound **4** (8.1 mg). Another active fraction from the hexane extract (fraction E, 27.3 mg) underwent ODS flash chromatography (12 × 250 mm), eluted with increasing MeOH (30−100%) in H_2O, to give compound **5** (2.0 mg).

GLI-Mediated Transcriptional Activity Assay. Detailed procedures were described previously.[12,13]

Cytotoxicity Test. Briefly, cancer cells (PANC1 and DU145) or normal cells (C3H10T1/2) were seeded onto 96-well black plates in 100 μL of RPMI medium containing 10% FBS at 1×10^4 cells per well and preincubated at 37 °C for 24 h. The medium was then replaced with fresh RPMI + 10% FBS containing different concentrations of each compound. 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. HaCaT-expressed exogenous GLI1 cells were seeded onto a 10 cm dish (2×10^6 cells/dish) and incubated for 12 h at 37 °C. To activate expression of exogenous GLI1 protein, 1 μg/mL of tetracycline was added into each well, followed by another 12 h incubation. DMSO medium (containing 5% FBS) was removed, and compounds **1**, **2**, and **5** in various concentrations were added. After 24 h incubation, cells were washed with PBS, then homogenized 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 1% proteasome inhibitor cocktail (Nacalai Tesque, Tokyo, Japan) and incubated on ice for 30 min. Supernatants of the cell lysates, as obtained from a 4 °C centrifugation (30 min), were subjected to a 5% and a 12.5% SDS-PAGE electrophoresis, then transferred to a polyvinylidine difluoride membrane (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 hybridized at room temperature for 1 h with primary antibodies: GLI1, PTCH (Santa Cruz) and BCL-2 (Sigma). Another 1 h incubation of blots-hybridized secondary antibodies was performed at room temperature. The secondary antibodies used were anti-goat IgG (Sigma), anti-rabbit IgG, and anti-mouse IgG (Amersham Biosciences). After washing with TBST, immunocomplexed bands were detected using an ECL Advance Western (GE Healthcare Biosciences) or an Immobilon Western (Millipore) detection system. To confirm protein expression level of PTCH and BCL-2 in PANC1, the same method as described above was performed.

Real-Time RT-PCR Analysis. Ptch mRNAs of PANC1 treated with compound **1** (2 and 4 μL), cyclopamine (0.2 and 0.4 μL), or control (no treatment) were reverse transcribed using a RT-PCR SuperScript III Platinum Two Step qRT-PCR kit (Invitrogen). The RT-PCR reactions were carried out on a Mx3000P QPCR system (Stratagene) with the following annealing temperatures: 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). Sequence primers were set as follows: GAPDH (5′-ATGGGGAAGGTGAAGGTCG-3′ and 5′-TAAAAGCAGCCCTG-GTGACC-3′); PTCH (5′-TCCTCGTGTGCGCTGTCTTCCTTC-3′ and 5′-CGTCAGAAAGGCCAAAGCAACGTGA-3′).

A standard curve was recorded, and the amount of target mRNAs in each sample was normalized with that of mean GAPDH.

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