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New Hedgehog/GLI signaling inhibitors from *Excoecaria agallocha*

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ABSTRACT

The inhibition of the Hedgehog (Hh) signaling pathway has emerged as an anti-cancer strategy. Three flavonoid glycosides including 2 new compounds (**1**–**2**) were isolated from *Excoecaria agallocha* as Hedgehog/GLI1-mediated transcriptional inhibitors and exhibited cytotoxicity against human pancreatic (PANC1) and prostate (DU145) cancer cells. Our data revealed that compound **1** clearly inhibited the expression of GLI-related proteins (PTCH and BCL-2) and blocked the translocation of GLI1 transcription factors into the nucleus in PANC1. Deleting the Smoothened (Smo) function in PANC1 treated with **1** led to downregulation of the mRNA expression of Ptch. This study describes the first Hh signaling inhibitor which blocks GLI1 movement into the nucleus without interfering with Smo.

The Hedgehog signaling pathway is critical for cell growth and stem cell maintenance. Damage to essential core components of the Hh pathway often results in congenital birth defects[1] whereas an aberrant activated Hh pathway leads to cancer. In cancer cells, binding of the Hh protein ligand to receptor patched 1 (PTCH) abolishes the inhibitory effect of PTCH on proto-oncogene Smoothened (Smo), thereby allowing Smo to activate the GLI family of transcription factors.[2]

In recent reports, three sites have been mainly targeted to identify Hh-signaling inhibitors: Smo protein (cyclopamine,[3a,b] SANTs,[3c] and CUR61414[3d]), GLI protein (GANTs[3e]) and Hh ligand (robotnikinin[3f]). Several studies have suggested that GLI1 is one of the critical transcriptional effectors closely associated with tumor formation by direct association with a specific binding site (5′-GACCACCCA-3′) in the promoter region of the target genes.[4]

The regulation of GLI1 in mediating oncogenic Hh signaling is poorly known. Some reports describe that activated mutations of Smo induce the nuclear translocation of transcriptional GLI factors.[5a,b] The use of small molecule inhibitors of Smo showed potentially limited efficacy. These inhibitors only treat tumors where pathway activation has occurred upstream or at the level of Smo, yet cancer with other downstream components is unresponsive to Smo inhibitors.[5c] Therefore, searching for small molecule inhibitors that target GLI1 in an Smo-independent manner will be of great importance in response to the urgent need for broadly active downstream inhibitors of Hh signaling.

We have recently reported naturally occurring Hh/GLI-mediated transcriptional inhibitors.[6a–c] This Letter reports the structural elucidation of new Hh/GLI1-mediated transcriptional inhibitors and demonstrates suppression of the protein level and mRNA expression by the most potent inhibitor (compound **1**), isolated from *Excoecaria agallocha* leaves.

The MeOH extract of dried leaves of *E. agallocha* was partitioned between hexane, EtOAc and BuOH, followed by repeated column chromatography and preparative HPLC to yield compounds **1**–**8**, consisted of two new compounds (**1** and **2**), and 6 known flavonoid glycosides (**3**–**8**) (Fig. 1); afzelin (**3**),[7a] quercitrin (**4**),[7b] rutin (**5**),[7c] kaempferol-3-*O*-(2-*O*-acetyl-α-L-rhamnopyranoside (**6**),[7d] kaempferide 3-*O*-α-L-rhamnopyranoside (**7**),[7e] and kaempferol 3-*O*-α-L-arabinofuranoside (**8**).[7f] The structure elucidation of known compounds was on the basis of comparison with spectral data of the reported values (Table 1).

Compound **1** was obtained as a yellow powder and had a molecular weight at *m/z* 485.1162 ([M+Na]$^+$, Δ −2.1 mmu), corresponding to the molecular formula of $C_{22}H_{22}O_{11}$ in the HR-FABMS. The IR absorption bands suggested the presence of hydroxyl (3317 cm^{-1}) (br) and carbonyl (1680 cm^{-1}) groups. The UV absorption maxima were at UV (MeOH) λ_{max} 261 nm (log ε 3.8) and 355 nm (log ε 3.0). Acid hydrolysis of compound **1** gave quercetin and acofriose (3-*O*-methyl rhamnose). Acofriose was determined as having an L-form by comparison of its specific rotation $[\alpha]_D^{22}$ +35 (*c* 0.1, MeOH) with those reported in the literature ($[\alpha]_D^{25}$ +39).[8] The carbon signals at δ_C 177.7 (C-4), 148.4 (C-3′) and 145.2 (C-4′) were in agreement with the quercetin skeleton bearing a carbonyl and two hydroxyl groups. In addition, four signals of OH hydrogens were observed at δ_H 12.63 (1H, s), 10.88 (1H, s), 9.71 (1H, s), and 9.34 (1H, s).

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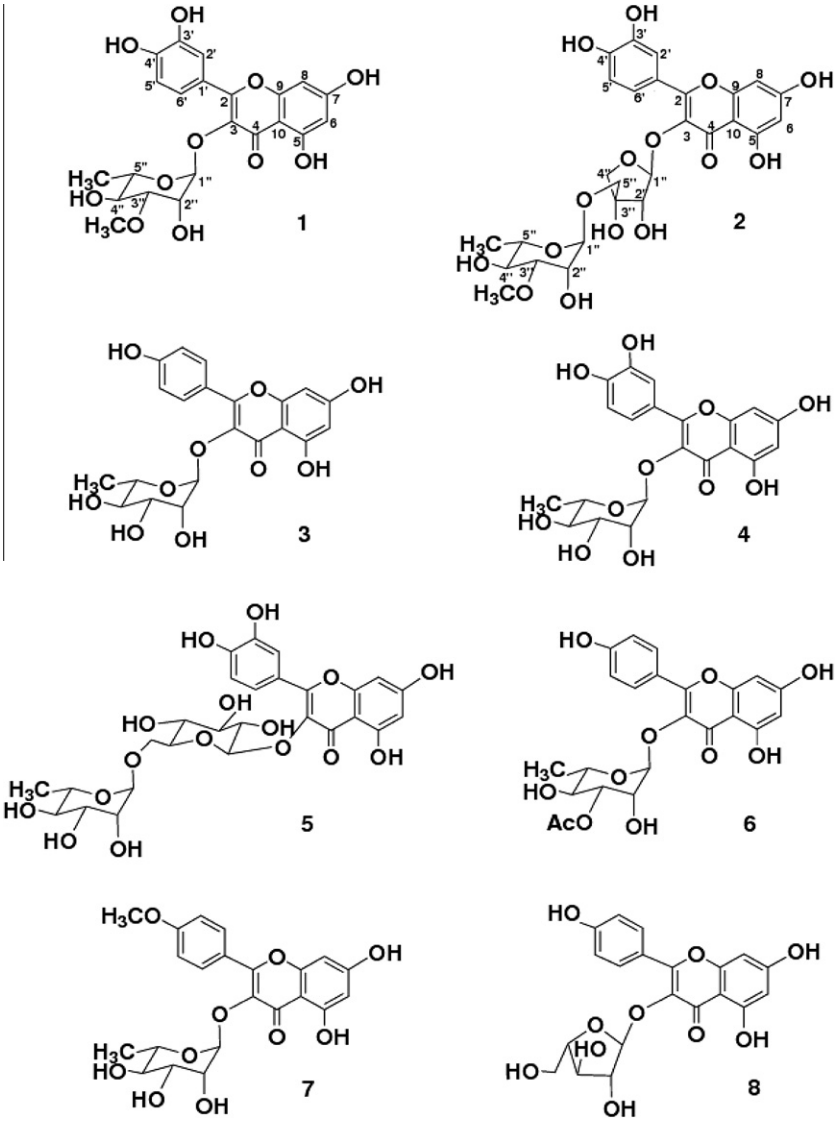

Figure 1. Chemical structures of compounds **1**–**8** from *E. agallocha.*

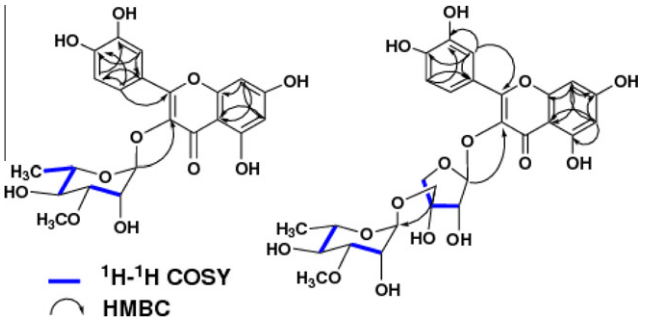

Figure 2. Key ^{1}H–^{1}H COSY and HMBC correlations observed for **1** and **2**.

The location of the sugar part, according to HMBC analysis (Fig. 2), was shown to attach to the C-3 of aglycone. The small coupling constant (1.6 Hz) of the anomeric proton (δ_H 5.24) indicated its α configuration.[9]

Compound **2** was isolated as a yellow amorphous solid and gave the molecular formula $C_{27}H_{30}O_{15}$, as deduced from HR-FABMS *m/z* 617.1584 (calcd for $C_{27}H_{30}O_{15}Na$, 617.1558). The proton signals showed two aromatic doublets at δ_H 6.20 and 6.39, and an ABX system at δ_H 7.66 (J = 2 Hz), 6.84 (J = 8 Hz), and 7.56 (J = 2 Hz and 8 Hz), confirming that the aglycone was quercetin. The ^{13}C NMR spectrum of **2** showed 27 resonances, including one methoxy group (δ_C 48.5) attached to one of the sugar moieties. Comparison of NMR spectral data of **2** differed from **1** in the sugar chain. Further HMBC analysis (Fig. 2) indicated the connections of sugar units between δ_H 5.46 (H-1″) and δ_C 133.4 (C-3), and between δ_H 3.63 (H-5″) and δ_C 101.7 (C-1‴). Acid hydrolysis and HPLC isolation gave quercetin, D-apiose and L-acofriose. The orientation of the anomeric proton of the first sugar (D-apiose, δ 5.46) was deduced as β since the coupling constant was smaller (J = 3.3 Hz)[10] than that of α (J = 4.5 Hz).[11] Furthermore, the small coupling constant observed for the anomeric proton of the second sugar (L-acofriose, δ 5.37, J = 2.0 Hz) suggested its α configuration.[9] The D-form of the first sugar connected to aglycone was confirmed by comparing its specific rotation $[\alpha]_D^{21}$ +6.1 (c 0.03, H_2O) with the reported values of an authentic sample, $[\alpha]_D$ +5.2 (c 1.1, H_2O).[12] The downfield location of the C-3‴ absorption of the second sugar showed the presence of a methoxy group at C-3‴. The sugar was identified as 3-*O*-methyl-rhamnose and was confirmed as having an L-form by the same method as compound **1**.

Hh/GLI1 inhibitory effects of all isolated compounds (**1**–**8**) were further evaluated. Compounds **1**, **2** and **8** inhibited

Table 1
^{1}H and ^{13}C NMR spectral data of **1** and **2**

Position	1		2	
	δ_H (J in Hz)[a]	δ_C[a]	δ_H (J in Hz)[a]	δ_C[a]
2		156.4		156.3
3		134.2		133.4
4		177.7		177.5
5		161.3		161.2
6	6.19 d (2.0)	98.6	6.20 d (2.0)	98.6
7		157.3		156.2
8	6.38 d (2.0)	93.6	6.39 d (2.0)	93.5
9		156.4		156.1
10		104.1		103.9
1′		120.7		121.6
2′	7.28 d (2.0)	115.6	7.66 d (2.0)	116.2
3′		148.4		148.4
4′		145.2		144.8
5′	6.86 d (8.0)	115.4	6.84 d (8.0)	115.2
6′	7.25 dd (2.0, 8.0)	121.1	7.56 dd (2.0, 8.0)	121.1
1″	5.24 d (1.6)	101.8	5.46 d (3.3)	100.8
2″	4.92 dd (1.6, 3.0)	70.5	4.44 d (3.3)	74.0
3″	3.12 dd (9.0, 3.0)	71.1	—	76.5
4″	3.50 d (9.0)	70.0	3.57 d (9.6)	77.5
			3.57 d (9.6)	
5″	4.70 m	70.3	4.02 d (8.0)	67.9
			3.63 d (8.0)	
6″	0.79 d (6.0)	17.5		
1‴			5.37 d (2.0)	101.7
2‴			5.07 dd (2.0, 1.2)	71.1
3‴			3.07 dd (8.0, 1.2)	73.2
4‴			3.20 d (8.0)	69.9
5″			4.95 m	75.8
6″			0.79 d (6.0)	17.5
4‴-OCH_3	3.15 s	48.6	3.16 s	48.5
5-OH	12.63 s			
7-OH	10.88 s			
3′-OH	9.34 s			
4′-OH	9.71 s			

[a] In DMSO-d_6.

Hh/GLI-mediated transcriptional activity with IC_{50} values of 0.5, 19.1 and 2.0 μM, respectively, whereas compounds **3**–**7** were inactive (Fig. 3A, Table 2). In this assay, Gli1 protein expression was induced by 12-h treatment with tetracycline. Removal of tetracycline after another 12 h, followed by the addition of samples eliminated the undesired production of GLI1 via CMV promoter activation. Cell viability of compounds was checked simultaneously with luciferase activity under a fluorimetric microculture cytotoxicity assay (FMCA) system.[13] Of the 3 active compounds, compound **1** appeared to be the most potent Hh/GLI-1 inhibitor.

We further examined the cytotoxicity of active compounds against PANC1, DU145 and C3H10T1/2 using the FMCA system. The results revealed that compounds **1**, **2**, and **8** were cytotoxic against PANC1 cells (IC_{50} values of 0.7, 20.0 and 1.8 μM, respectively) and DU145 cells (IC_{50} values of 0.8, 22.0 and 2.4 μM, respectively) but did not affect normal cell lines (Fig. 3B, Table 2). The cytotoxicity of **1**, **2**, and **8** against PANC1 and DU145 cells may be associated with their Hh/GLI transcriptional activity inhibition.

To ensure that the inhibition of Hh signaling by **1** was associated with the expression of GLI-related proteins (PTCH and BCL-2) and to identify the effect of **1** on downstream events, we checked the protein levels of full, cytoplasmic and nuclear PANC1 after treatment with **1**. As a result, **1** reduced the expression of PTCH and BCL-2 proteins in a dose-dependent manner. Our Western blot result also showed that treatment with **1** at a concentration of 1.6 μM led to a significant decrease in the protein level of nuclear GLI1 in PANC1 (Fig. 4A).

To further understand the molecular mechanism underlying the Hh signaling inhibitory effect of **1**, we checked the expression of a GLI-related gene (Ptch) using real-time quantitative RT-PCR. It has previously been suggested that Ptch is a repressive Hh receptor, and elevated expression of this gene results in the concomitant expression of Hh target genes, including GLI1.[3e] Consistent with this, our data showed that the downregulation of Ptch mRNA expression is essential for the inhibition of Hh/GLI1 signaling on PANC1 treated with **1** (Fig. 4B).

Loss of Ptch function has been reported to cause aberrant Hh signaling by Smo.[14] Most known Hh modulators, including cyclopamine, repress the pathway by antagonizing Smo activation; however, one of the oncogenic Smo mutants (SmoM2) is apparently resistant to cyclopamine[15] and most Smo inhibitors were not effective against medullablastoma and cancer associated with downstream lesions.[16]

To verify the function of Smo in PANC1 during the Hh inhibition of **1**, we knocked down Smo expression in PANC1 cells by siRNA and performed real-time quantitative RT-PCR on its mRNA expression. Transfection of a non-targeting siRNA at the same concentration served as a control. The transfection results, as confirmed by

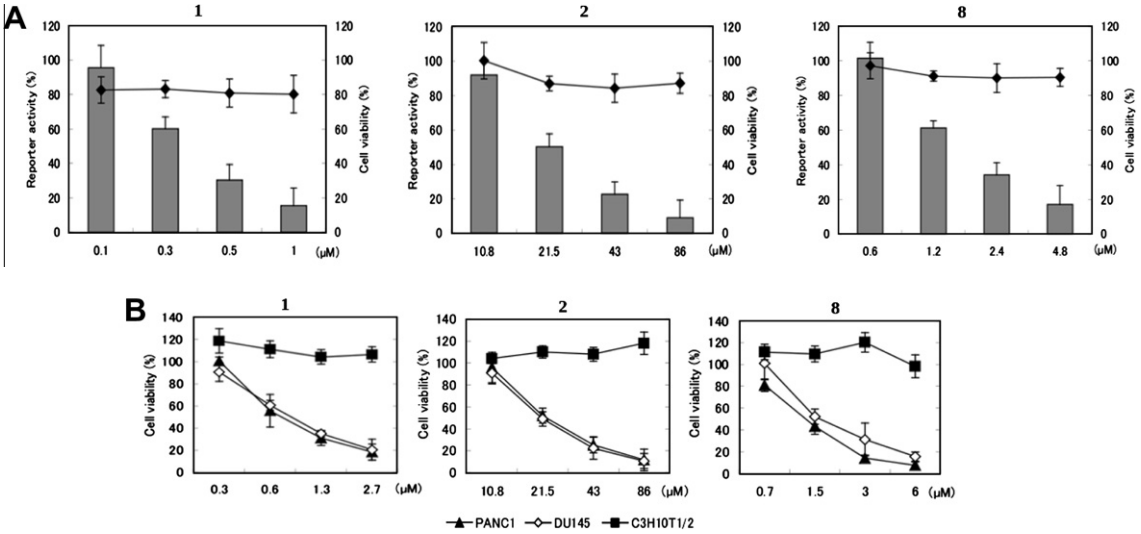

Figure 3. (A) Inhibition of GLI1-mediated transcriptional activity (solid columns) and cell viability (solid curves) of compounds **1**, **2**, and **8**. HaCaT-GLI1-Luc cells were seeded onto a 96-well plate (2×10^5 cells per well) and then treated with compounds 12 h after tetracycline addition. Cell viability and luciferase activity were determined at the same time. (B) Cytotoxicity of compounds **1, 2**, and **8** against PANC1, DU145, and C3H10T1/2 cells. Assays were performed at 0.05% DMSO ($n = 3$). Error bars represent sd.

Table 2
IC_{50} values (μM) of GLI-mediated transcriptional inhibition and cytotoxicity against cancer cells (PANC1, DU145) and C3H10T1/2 cells

Compound	GLI transcriptional inhibition (IC_{50}, μM)	Cytotoxicity (IC_{50}, μM)		
		PANC1	DU145	C3H10T1/2
1	0.5	0.7	0.8	>100
2	19.1	20.0	22.0	>100
8	2.0	1.8	2.4	>100

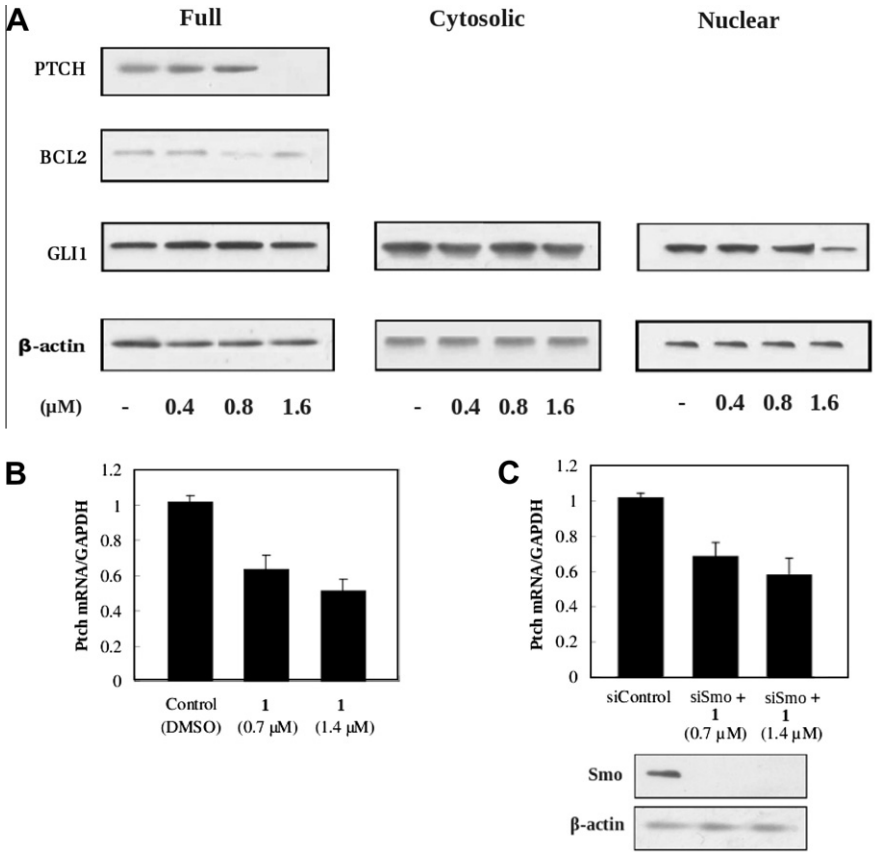

Figure 4. (A) Effect of compound **1** on GLI1 and/or GLI-related protein (PTCH and BCL2) levels in full, cytosolic and nuclear PANC1 cells. (B) Inhibition of Ptch mRNA expression by compound **1** in PANC1 cells. GAPDH was used as an internal control. Assays were performed at 0.05% DMSO ($n = 3$). Error bars represent sd. (C) Expression of Ptch mRNA (upper panel) and Smo protein (bottom panel) in PANC1 treated with **1** after siRNA-mediated silencing of Smo.

Western blotting, proved the total depletion of the Smo protein level after a silencing process (Fig. 4C, bottom panel). Accordingly, silencing of Smo siRNA significantly reduced the expression of Ptch mRNA expression in PANC1 cells treated with **1** (Fig. 4C, upper panel). These results thus indicated that **1** inhibited Hh signaling in an Smo-independent manner.

Because the inhibitory effect of **1** resulted in the decreased level of GLI1-related proteins (PTCH and BCL-2) as well as blocking GLI1 transcription factor translocation into the nucleus in PANC1 cells (Fig. 4A), we may speculate that this compound antagonizes GLI1 activator function. In accordance with this result, several reports have previously described that GLI function can be modulated without Smo interference via numerous types of GLI signaling, such as transforming growth factor-β (TGFβ),[17a] mitogen-activated protein kinase (MAPK)[17b] and posphatidylinositol 3-kinase (PI3K)/Akt signaling.[17c]

In conclusion, we have isolated naturally occurring Hh/GLI inhibitors including 2 new compounds (**1**–**2**) from *E. agallocha*. Compound **1** inhibited the translocation of GLI1 transcription factor into the nucleus of PANC1. Quantitative RT PCR of **1** exhibited that the reduction of Hh signaling was not caused by Smo interference, but rather by other unrevealed mechanisms related to the GLI1 inhibitory function. This is the first report of an Hh/GLI signaling inhibitor which reduces the level of GLI1 in the nucleus.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.126.

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