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Naturally Occurring Small-Molecule Inhibitors of Hedgehog/GLI-Mediated Transcription

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*The aberrant hedgehog (Hh)/GLI signaling pathway causes the formation and progression of a variety of tumors. To search for Hh/GLI inhibitors, we screened for naturally occurring inhibitors of the transcriptional activator GLI1 by using a cell-based assay. We identified zerumbone (**1**), zerumbone epoxide (**2**), staurosporinone (**9**), 6-hydroxystaurosporinone (**10**), arcyriaflavin C (**11**) and 5,6-dihydroxyarcyriaflavin A (**12**) as inhibitors of GLI-mediated transcription. In addition, we isolated physalins F (**17**) and B (**18**) from* Physalis minima*, which are also potent inhibitors. These compounds also inhibited GLI2-mediated transactivation. Semi-quantitative RT-PCR and Western blotting analysis further revealed that **1**, **9**, **17**, and **18** decreased Hh-related component expressions. We also show that inhibitors of GLI-mediated transactivation reduce the level of the antiapoptosis Bcl2 expression. Finally, these identified compounds were cytotoxic to PANC1 pancreatic cancer cells, which express Hh/GLI components. These results strongly suggest that the cytotoxicity of the compounds to PANC1 cells correlates with their inhibition of GLI-mediated transcription.*

Introduction

The hedgehog (Hh)/GLI signaling pathway plays an important role in a variety of aspects of vertebrate development, including pattern formation and proliferation, and differentiation of a number of different cell types. The vertebrate hedgehog family includes three members: sonic hedgehog (Shh), desert hedgehog, and indian hedgehog. Shh is the most extensively characterized vertebrate homologue and is important during embryonic development, particularly for the regulation of pattern formation and induction of cell proliferation in numerous tissues.[1–3]

Hh signal transduction is initiated by the induction of the Hh precursor protein (45 kDa) in Hh-sending cells, after which the precursor undergoes autocatalytic processing and modification. The precursor is cleaved to a 20 kDa N-terminal signaling domain and a 25 kDa C-terminal catalytic domain.[4] After autoprocessing of the precursor, a cholesterol molecule is bound covalently to the carboxy terminus of the N-terminal domain. The modified N-terminal signaling domain is secreted into the cytosol as a Hh ligand. On the Hh-receiving cells, Patched (Ptch), a twelve-pass transmembrane protein, interacts with the Hh ligand and Smoothened (Smo), a seven-pass transmembrane protein that is a signal transducer.[5] In the absence of Hh ligand, Ptch interacts with Smo to inhibit its function and prevent activation of the downstream signaling cascade. Once the Hh ligand binds to Ptch along with Hh-interacting protein, Smo inhibition is released; this results in the activation of a downstream signaling cascade. This causes the release of the transcriptional factor GLI from a macromolecular complex on microtubules that includes the suppressor of fused, fused, protein kinase A, GLI and possibly other components.[6]

GLIs, which are zinc-finger transcription factors participate in the final step of the Hh/GLI signaling pathway, and they regulate several genes, including those that are related to cell cycle control and Hh/GLI signaling.[7] GLI1 acts as a transcriptional activator, whereas GLI2 and GLI3 act as both activators and repressors.[8] All GLIs bind to DNA through five zinc-finger domains that recognize the consensus GLI-selective sequence 5′-GACCACCCA-3′, which regulates transcription.[9,10]

Besides its crucial roles in development, aberrant Hh/GLI signaling in adult tissues has recently been implicated in cancer formation and development[11,12] in the skin,[13] brain,[14] prostate,[15] upper gastrointestinal tract,[16] pancreas[17] and lung.[18] In several types of human tumors, such as basal cell carcinoma and medulloblastoma, Hh/GLI signaling is constitutively activated due to mutations in Ptch or Smo; this leads to GLI-mediated transcriptional activation and therefore tumor formation and progression.[19] Thus, interfering with GLI function, which is the last step in Hh/GLI signaling might be a good target for the development of therapies for treating cancers that are caused by mutations in Smo or Ptch.

Natural products have played a prominent role in drug discovery for many years.[20] We have previously isolated a variety of naturally occurring bioactive compounds from tropical

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plants, myxomycetes (true slime molds) and seaweed.[21,22] Cyclopamine and jervine are natural steroidal alkaloids from plants of the genus *Veratrum* that block the Hh signaling pathway by antagonizing Smo function.[23] Other Hh signaling antagonists, in fact, are currently entering clinical trials. In addition, synthetic inhibitors of GLI-mediated transcription have been reported,[24,25] but there is still an urgent need to find potent inhibitors of this transcription factor.

Here, we constructed a cell-based reporter assay of GLI-mediated transcription to search for natural products that inhibit GLI. We identified several types of active compounds from natural products and plant extracts libraries. Finally, we determined the effect of these inhibitors on protein expression.

Results and Discussion

Construction of a cell-based assay of GLI1-mediated transcription

To find specific inhibitors of Hh/GLI signaling pathway from a compound library and natural resources, we constructed a cell-based reporter assay for measuring the transcriptional activity of GLI1, which constitutes the final step in the Hh signaling pathway. We targeted the GLI-mediated transcription, which is the last step of the Hh/GLI signaling pathway. Although Regal et al.[26] previously established an assay by using HaCaT cells that express GLI1 or GLI2 under tetracycline control (T-REx system), we altered this system to make it simpler and more sensitive. We began with a new reporter vector that has 12 consecutive GLI binding sites (12×GACCACCCA), a TK promoter, and a luciferase gene (Figure 1). The 12 consecutive GLI binding sites and the TK promoter were inserted into the pGL4.20 plasmid (Promega) via the NheI/BglII restriction sites. The constructed plasmid, pGL4-GLI BS, was transiently transfected into HaCaT cells that express exogenous GLI1 protein under tetracycline control. The luciferase activity in the presence of tetracycline (+Tc) was 12.3-fold higher than in its absence (−Tc; Figure 2A). This indicates that an increase of reporter activity depends on the production of endogenous GLI1 protein. Thus, a decrease of reporter activity by samples shows the inhibition of GLI1-mediated transcription. For construction of a stable expression system, cells that were stably transfected with pGL4-GLI BS were selected with puromycin. The luciferase activities were measured for all of the transfectants, and one of them showed a 9.3-fold difference (+Tc/−Tc) (Figure 2B). This transfectant (HaCaT-GLI1-luc) was selected for the screening of compounds and natural resources.

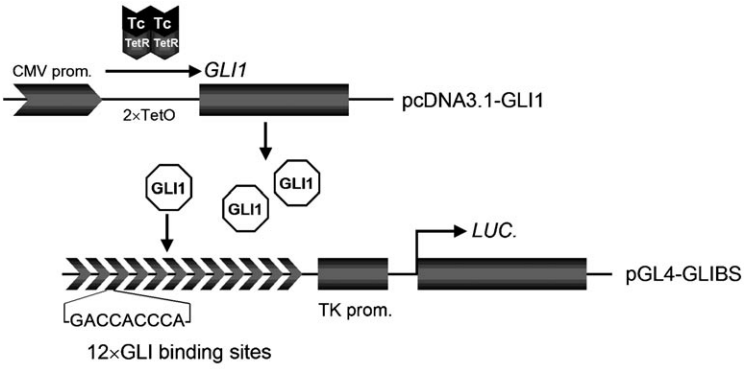


Figure 1. The constructed assay system. pcDNA3.1-GLI1 expresses GLI1 protein by a tetracycline-regulated CMV promoter, and GLI1 protein binds onto GLI binding sites of pGL4-GLI BS. Tetracycline removes TetR to start GLI1 protein expression (T-REx system). Tc, tetracycline; TetR, tetracycline repressor; TetO, tetracycline operator; CMV, human cytomegalovirus promoter; GLI1, transcription factor of Hh/GLI signaling pathway.

Next, we optimized the screening protocol, including the cell numbers, time of incubation with tetracycline, and the time of incubation with test samples. The expression level of GLI1 protein was assessed by Western blotting at 6, 12, and 18 h after the addition of tetracycline (Figure 2C). We found that sufficient levels of exogenous GLI1 protein were expressed after 6 h. For screening in 24 or 96-well plates, we measured the luciferase activity of 0.2, 0.5, 1 and 2×10^5 cells per well at 6, 12, 24, 30, and 36 h after the addition of tetracycline, and we calculated the ratio of activity compared to that in the absence of tetracycline (+Tc/−Tc; Figure 2D). The level of GLI1 protein and the luciferase activity nearly reached a plateau after 24 h at all cell densities except for 2.0×10^5 cells per well. To determine the effects of the inhibitors, we measured the activity of test samples between 12 and 24 h after the addition of tetracycline because the effect of compounds on transactivation of luciferase cannot be detected clearly at the plateau point of luciferase expression.

This assay could detect not only GLI1-mediated transcriptional inhibitors but also inhibitors of CMV promoter activation if both the sample and tetracycline were incubated at the same time. Indeed, we found that two natural products from the methanol extract of *Cassia alata*, kaempferol and naringenin, reduced the luciferase reporter activity, but their effects were due to the inhibition of the CMV promoter activity of the GLI1 expression vector, pcDNA3.1-GLI1 (Figure 2E). This finding was further confirmed by the fact that kaempferol reduced the luciferase activity in a transient transfection experiment by using pCMV-luc (data not shown). To avoid selecting inhibitors of the CMV promoter, it is essential that there is a sufficient amount of GLI1 protein in the cells at the time of sample addition. Figure 2F shows the time courses of luciferase activity under two conditions: removal of tetracycline (+Tc→−Tc) and non-removal of tetracycline (+Tc→+Tc) after a 12 h treatment with tetracycline. The luciferase activity of the +Tc→−Tc condition remained a constant value for 24 h after, whereas the activity in the +Tc→+Tc condition increased due to continued GLI1 production. The production of GLI1 via the CMV promoter was almost halted by removing tetracycline. Thus, by removing tetracycline (+Tc→−Tc), the contribution of the CMV promoter to the effects of the test sample can be essentially eliminated. Thus, the assay protocol was as follows. First, GLI1 protein expression was induced by a 12 h treatment with tetracycline. Tetracycline was then removed. Finally, the cells were treated with test samples for 12 h.

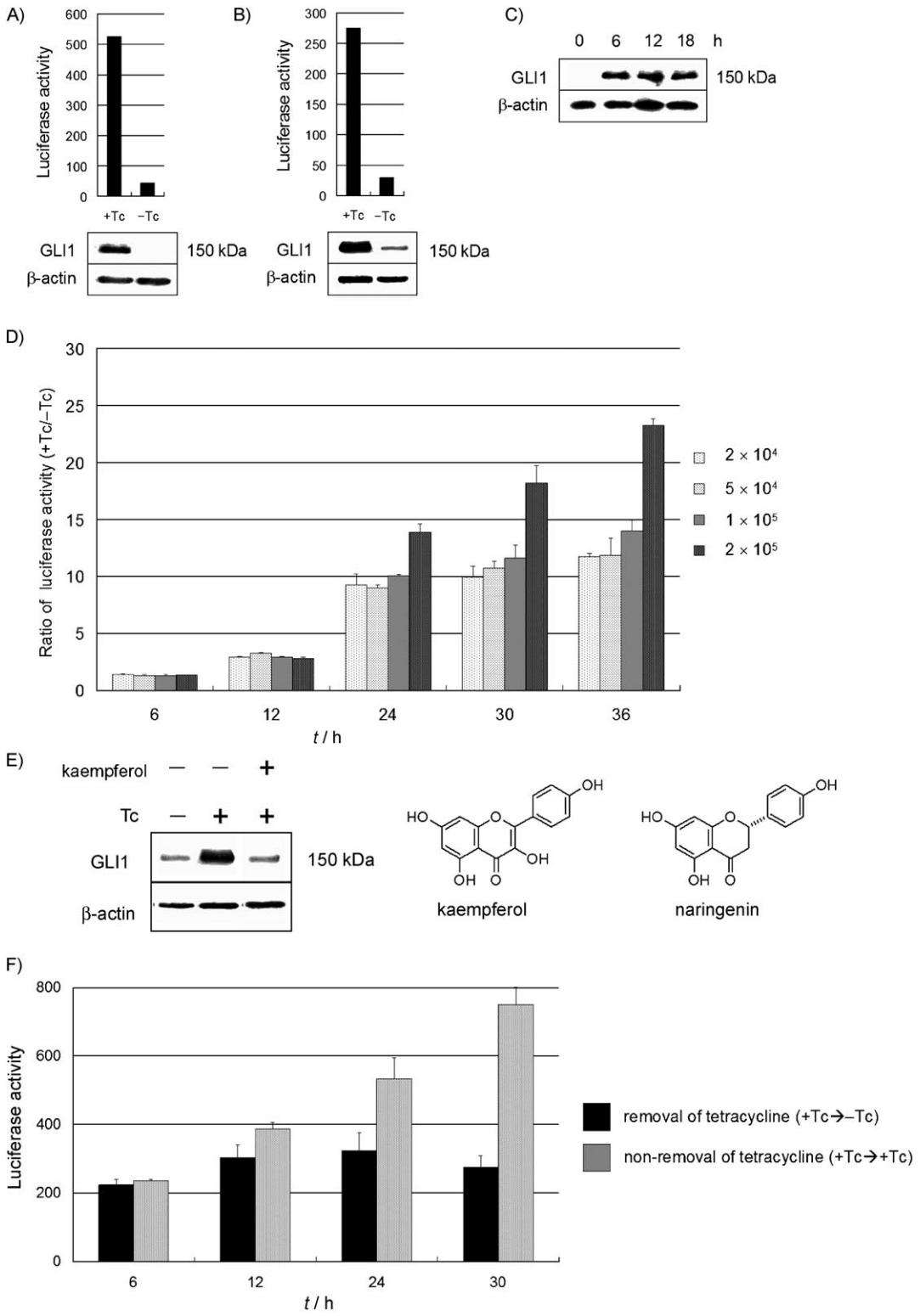
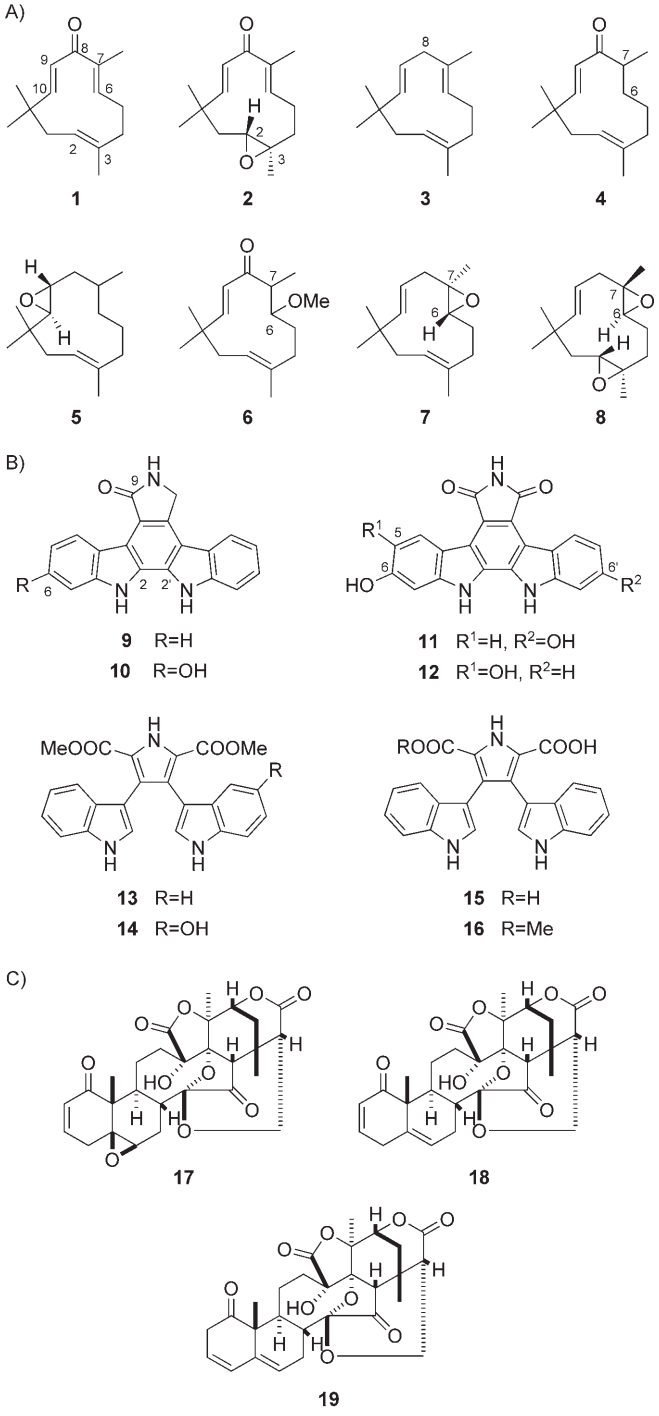


Figure 2. Analysis of the screening system. A) Constructed pGL4-GLI BS was transiently transfected into HaCaT cells that express exogenous GLI1 protein by regulation with tetracycline. GLI1 induction by tetracycline addition increased luciferase activity at 24 h after the addition of tetracycline. −Tc, untreated with tetraycyline. B) HaCaT cells stably transfected with pGL4-GLI BS were treated with tetracycline for 24 h to induce exogenous GLI1 protein and luciferase activity. C) GLI1 protein inductions at each time of 0, 6, 12, and 18 h after the addition of tetracycline were examined with Western blotting analysis. D) HaCaT-GLI1-luc cells of 0.2, 0.5, 1 and 2×10^5 cells per well were treated with tetracycline, and the luciferase activity was measured at each time (6, 12, 24, 30 and 36 h). All of the measurements of luciferase activity were performed in triplicate, and data were shown as the mean s.d. E) Kaempferol inhibited the exogenous GLI1 expression by disturbing CMV promoter activity. Cells (HaCaT-GLI1-luc) were treated with kaempferol and tetracycline for 24 h, and then exogenous GLI1 expression was checked by Western blotting analysis. F) HaCaT-GLI1-luc cells were treated with tetracycline for 12 h. After removing tetracycline by washing with PBS, the cells were incubated in tetracycline-free medium (■) or in tetracycline-containing medium (■) for 6, 12, 24 and 30 h. At each time, the luciferase activity was measured in triplicate, and the data are shown as the mean s.d.

It is also possible that a decrease in luciferase reporter activity in this assay is due to the cytotoxicity of the test samples. We therefore also examined the cytotoxicity of samples by using a fluorimetric microculture cytotoxicity assay (FMCA).[27] We designated samples that exhibit both low luciferase reporter activity and high cell viability as "positive", which means that they are inhibitors of GLI1-mediated transcription.



Scheme 1. The chemical structures of identified natural products that inhibit GLI-mediated transcription and the related compounds. A) Sesquiterpenes **1**–**8**. B) Bisindole alkaloids **9**–**16**. C) Physalins **17**–**19**.

To elucidate that our assay system targets downstream of Smo, cyclopamine, a Smo antagonist, was tested, and as we expected, cyclopamine did not decrease the reporter activity at 40 μM in our system. Because of overexpressed GLI1, the inhibitors that were found in this screening system most probably act downstream of GLI. Thus, our assay system would find Hh/GLI inhibitors that are different from Smo antagonists such as cyclopamine.

Screening of our natural products library

By using our cell-based assay system, the screening was performed with 94 compounds from our natural product library, including terpenoids, flavonoids, phenylpropanoids, their glycosides and bisindole alkaloids at concentrations of 25 and 2.5 μg mL^{-1}. We identified two sesquiterpenes and four bisindole alkaloids as inhibitors of GLI-mediated transcription (Scheme 1 A and B; Table 1). Sesquiterpenes zerumbone (**1**)[28]

Table 1. IC_{50} values of GLI1 and GLI2 transcriptional inhibitory activity.

Compound	GLI1[a]	GLI2[a]
1	7.1 (1.5)	0.91 (0.23)
2	55 (13.0)	90 (21.3)
9	1.8 (0.57)	2.7 (0.84)
10	3.6 (1.18)	n.d.
11	11.3 (4.0)	n.d.
12	6.9 (2.5)	n.d.
17	0.66 (0.35)	1.4 (0.80)
18	0.62 (0.32)	1.5 (0.80)
cyclopamine	> 40	

[a] μM (μg mL^{-1}), n.d. not determined.

and zerumbone epoxide (**2**)[29] inhibited the transcriptional activity of GLI1 with IC_{50} values of 7.1 and 55 μM, respectively, whereas humulene (**3**),[30] buddledone A (**4**),[31] humulene epoxide-III (**5**),[32] 6-methoxy-(2*E*,9*E*)-humuladien-8-one (**6**),[33] humulene epoxide-II (**7**),[34] and humulene 2,3;6,7-diepoxide (**8**),[35] which have the same chemical skeletons as zerumbone, were inactive even at 25 μg mL^{-1}. Bisindole alkaloids staurosporinone (**9**),[36] 6-hydroxystaurosporinone (**10**),[36] arcryaflavin C (**11**),[37] and 5,6-dihydroxyarcyriaflavin A (**12**)[36] also inhibited the transcriptional activity of GLI1, with IC_{50} values of 1.8, 3.6, 11.3 and 6.9 μM, respectively, whereas lycogarubin C (**13**),[38] lycogarubin B (**14**),[38] lycogarubic acid A (**15**),[39] and lycogarubic acid methyl ester (**16**)[40] were inactive even at 25 μg mL^{-1}. Compounds **1**, **2**, **9**, **10**, **11**, and **12** dose-dependently inhibited GLI1-mediated transcription, but had little effect on cell viability at concentrations that inhibited GLI1-mediated transcription (Figure 3 A–F).

Screening of plant extracts and isolation of naturally occurring compounds that inhibit GLI-mediated transcription

We next screened our library of 192 tropical plant extracts at 100 and 50 μg mL^{-1}, and the potently cytotoxic extracts were

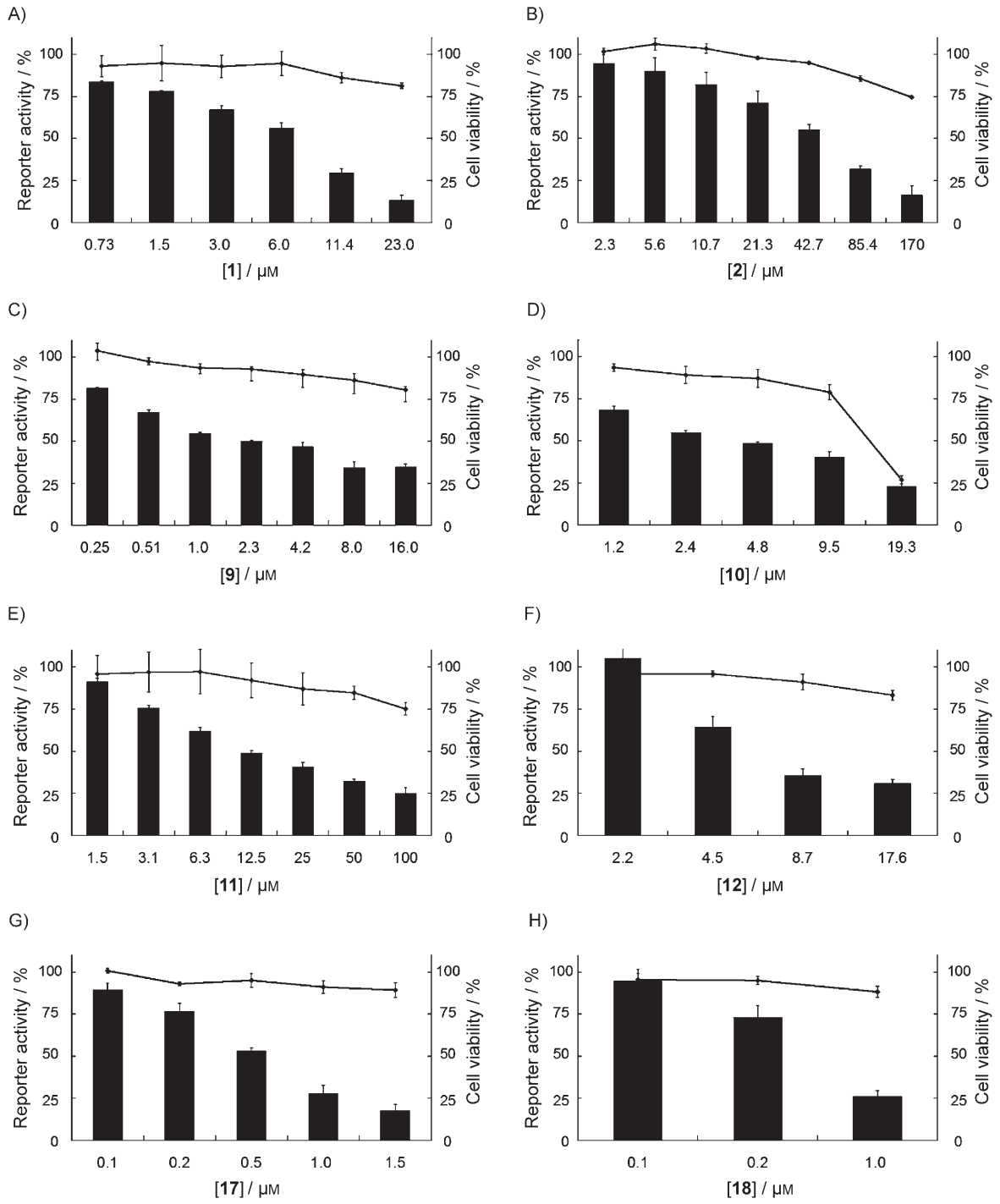


Figure 3. Inhibitors that were identified from the screening showed GLI1-mediated transcriptional inhibitory activity (solid column) in a dose-dependent manner with high cell viability (solid curves). Compounds were assayed in triplicate. All compounds were added as DMSO solutions. Bars, s.d.

again screened at lower concentrations (10 and 1 μg mL^{-1}). We found 15 extracts that dose-dependently reduced the reporter luciferase activity, but had little or no effect on cell viability. Of these extracts, the methanol extract of *Physalis minima* had the highest activity, with 34% reporter activity but 97% cell viability at 10 μg mL^{-1}. The methanol extract of *P. minima* (2.7 g) was partitioned between H_2O, and *n*-hexane and ethyl acetate. Cells that were treated with 10 μg mL^{-1} of the *n*-hexane and the ethyl acetate fractions showed 51% and 20% reporter activity and >95% cell viability. Because TLC analysis of the *n*-hexane and the ethyl-acetate-soluble fractions revealed the presence of nearly the same compounds, we combined and further purified them by consecutive steps of silica gel column chromatography, Sephadex LH-20 column chromatography and reversed-phase HPLC. This gave three compounds **17**, **18**, and **19** (Scheme 1C), which were identified as physalin F

(**17**),[41] physalin B (**18**),[42] and isophysalin B (**19**)[43] by comparing their spectral data with reported values. Physalins F (**17**) and B (**18**) dose-dependently inhibited GLI1-mediated transcriptional activity with little or no effect on cell viability (Figure 3G and H). The IC_{50} values of these two compounds in this assay were 0.66 μM (0.35 μg mL^{-1}) and 0.62 μM (0.32 μg mL^{-1}), respectively (Table 1). In addition, it should be emphasized that the compounds did not reduce the viability of HaCaT normal human keratinocytes near their IC_{50} concentrations.

There are three homologues of GLI in vertebrates: GLI1, GLI2 and GLI3.[8] GLI1 and GLI2 act as positive mediators of Hh/GLI signaling pathway in not only vertebrate development but also in tumor progression, and they share a high degree of homology. To examine whether the inhibitors of GLI1-mediated transcription also reduce GLI2-mediated transactivation, we transiently transfected pGL4-GLI BS into HaCaT cells that express GLI2 deleted of the N-terminal repressor domain (GLI2ΔN) under tetracycline control.[6,26] The transfectant was treated with tetracycline for 12 h to induce the expression of exogenous GLI2, followed by different concentrations of compounds **1**, **2**, **9**, **17**, and **18**, which inhibited GLI1-mediated transcription. All five compounds inhibited GLI2-mediated transcription, and their IC_{50} values were 7.1, 90, 2.7, 1.4, and 1.5 μM, respectively (Table 1). Thus, the relative potencies of the compounds for inhibition of GLI1 and GLI2-mediated transcription were similar.

Inhibition of GLI-mediated expression

During the screening of compounds that inhibit GLI1 and GLI2-mediated transcription, we identified three classes: sesquiterpenes **1** and **2**, bisindole alkaloids **9**, **10**, **11** and **12**, and 13,14-*seco*-16,24-cyclosteroids **17** and **18** (Scheme 1). We next investigated whether these compounds inhibit GLI-mediated protein expression. Because the expression of GLI1 and PTCH are known to depend on GLI-mediated transcription,[44] we investigated the effect of zerumbone (**1**), staurosporinone (**9**), physalin F (**17**) and physalin B (**18**) on their expression in HaCaT cells that express exogenous GLI1 or GLI2. First, we confirmed that GLI1 protein was expressed in HaCaT cells under tetracycline control by Western blotting. Sufficient GLI1 protein was expressed even after the addition of the compounds (Figure 4B); this confirms that the compounds do not inhibit the CMV promoter, and that assay system functioned correctly. We first investigated the effect of compounds **1**, **9**, **17** and **18** on the level of PTCH in GLI1-expressing HaCaT cells. Western blotting revealed that all of the compounds reduced the level of PTCH protein (Figure 4A).

Compounds **1**, **9**, **17** and **18** also inhibited GLI2-mediated transcription in a transient transfection experiment. We next investigated the effect of the compounds on the expression of GLI1 and PTCH proteins in HaCaT cells that express exogenous GLI2. GLI2 has been reported to induce GLI1 expression by binding to its promoter region.[45] In GLI2-expressing HaCaT cells under tetracycline control, the addition of tetracycline induced the expression of GLI1 and PTCH protein (Figure 4C). We found that compounds **1**, **9**, **17** and **18** caused a clear decrease in the expression of GLI1 and PTCH (Figure 4C).

The antiapoptosis protein Bcl2 is another target of the Hh/GLI signaling pathway. Previous studies have showed that transactivation of *bcl-2* is regulated by Hh/GLI signaling through GLI1[46] and GLI2.[47] These reports suggest that aberrant Hh/GLI signaling is related to the antiapoptotic character of cancer cells due to enhanced expression of Bcl2 protein. We therefore analyzed the effect of compounds **1**, **9**, **17** and **18** on the level of Bcl2 protein in HaCaT cells that express GLI1 or GLI2 under tetracycline control. In both exogenous GLI1 and GLI2-expressing cells, Bcl2 protein expression was increased when tetracycline was added, and the addition of compounds **1**, **9**, **17**, and **18** reduced the level of Bcl2 protein (Figure 4D and E). We further examined whether the inhibitors reduced the bcl2 promoter transcriptional activation by using PANC1 cells that express Hh/GLI signaling components. The pGL3-bcl2 reporter vector that possesses the *bcl2* 5′-flanking region with consensus 7×GLI binding sites was transiently transfected into PANC1 cells. After treatment of compounds **9**, **17**, **18** and cyclopamine, the luciferase reporter activities were reduced. The bcl2 promoter activity showed **9**, 61% at 16 μM; **17**, 74% at 3.8 μM; **18**, 68% at 3.9 μM; and cyclopmaine, 72% at 20 μM, respectively. These inhibitors of GLI-mediated transcription reduce the expression of the antiapoptotic protein Bcl2. This result also supports the reported relation between Hh antagonists and inhibition of Bcl2 expression.[47]

Zerumbone (**1**), which was isolated from *Zingiber zerumbet*, has been studied as a compound that causes cell death by inducing apoptosis.[48] This compound induces apoptosis in HepG2 cells by suppressing the expression of the antiapoptotic protein Bcl2, and by up-regulating the expression of the proapoptotic protein Bax; this results in an increase in the Bax/Bcl2 ratio.[49] Also, GLI binding sites have been reported in the *bcl2* 5′-flanking region.[46,47] Our findings suggest that the suppression of Bcl2 expression might be due to the inhibition of GLI-mediated transcription. Murakami et al.[50] examined the structure–activity relationship of zerumbone, and they found that an α,β-unsaturated carbonyl group might play a pivotal role in interactions with target molecules. Zerumbone (**1**) was the most potent inhibitor of GLI-mediated transcription of the sesquiterpenes (compounds **1**–**8**). Our results also show that the α,β-unsaturated carbonyl group in zerumbone is important for the inhibition of GLI-mediated transcription.

In our GLI-mediated transcriptional inhibitory assay, bisindole alkaloids **9**–**12** inhibited GLI-mediated expression, whereas compounds **13**–**16** did not. Thus, it appears that C2 and C2′ bonds in the structure of bisindole alkaloids are important for the inhibition of GLI-mediated transcription. Recent studies have showed that GLI transcription requires protein kinase C-δ (PKC-δ).[51] Because bisindole alkaloids have been reported as kinase inhibitors, compounds **9**–**12** could exhibit the inhibition of protein kinases that participate in GLI-mediated transactivation.

Physalins have been studied as cytotoxic compounds against many cancer cell lines.[52] Jacobo-Herrera et al. reported that physalins were modulators of the NF-κB cascade, and

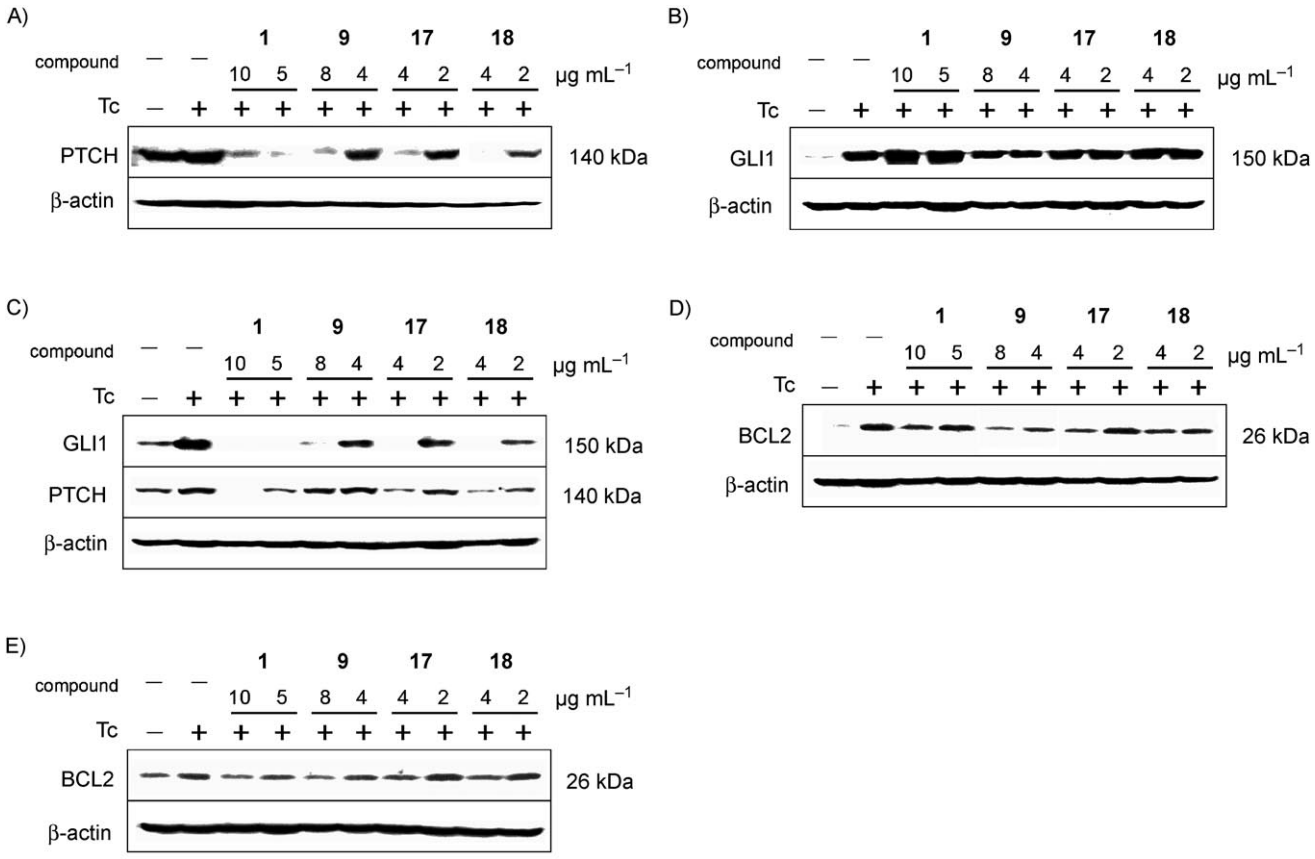


Figure 4. Evaluation of identified inhibitors in GLI-mediated transcriptional inhibition by Western blotting analysis. A) HaCaT cells that express tetracycline-regulated exogenous GLI1 were treated with tetracycline to induce exogenous GLI1 protein for 12 h. After treatment with the inhibitors **1**, **9**, **17** and **18** of each concentration for 24 h, PTCH protein expression was analyzed by Western blotting. B) Treatment with tetracycline induced exogenous GLI1 protein, and the inhibitors did not inhibit the exogenous GLI1 production. C) The HaCaT cells that express GLI2 protein were treated with the inhibitors after tetracycline addition, the production of GLI1 and PTCH proteins also decreased. D) and E) The effects on BCl2 protein expression in GLI1 (D) and GLI2-expressing cells (E) were examined by Western blotting analysis. The HaCaT cells that express exogenous GLI1 or GLI2 were treated with the inhibitors for 24 h, and the BCl2 expression was examined by Western blotting analysis. All of the experiments were performed twice. The HaCaT cells were treated with the inhibitors for 24 h. The proteins were separated with 7.5% (GLI1 and PTCH) and 12.5% (BCl2) SDS-PAGE. The proteins were detected by immunoblotting by using specific antibody. The β-actin was used as a control. The treatment concentrations (10 and 5 $\mu g\,mL^{-1}$ for **1**, 8 and 4 $\mu g\,mL^{-1}$ for **9**, 4 and 2 $\mu g\,mL^{-1}$ for **17**, and 4 and 2 $\mu g\,mL^{-1}$ for **18**) corresponded to 46 and 23 μM for **1**, 25 and 13 μM for **9**, 7.6 and 3.8 μM for **17**, and 7.8 and 3.9 μM for **18**, respectively.

showed inhibitory activity on PMA-induced NF-κB activation.[53] From theses reports, the mechanism of GLI-mediated transcriptional inhibition by physalins might include a path of PKC inhibition.

Compounds **1**, **9**, **17** and **18** have a different mechanism of action from cyclopamine, which is an antagonist of Smo, because cyclopamine did not decrease the reporter activity in our assay system. This is the first report that these natural products act as inhibitors of GLI-mediated transcriptional activity.

Effect of inhibitors on PANC1 cell

PANC1, a human pancreatic cancer cell line, expresses numerous Hh/GLI signaling pathway components, including Shh, Ptch, suppressor of fused, GLI1 and GLI2; this indicates that the Hh/GLI signaling pathway is activated in PANC1 cells. We confirmed the effect of compounds (**1**, **9**, **17**, and **18**) on the Hh/GLI signaling components' expression in PANC1 cells by semiquantitative RT-PCR analysis (Figure 5 A). These compounds decreased the mRNA expression of *Gli1*, *Gli2* and *Ptch* genes. These results indicate that the compounds inhibit the expression of these components at the transcriptional level. We further examined the effect of **17** and **18** on the protein expression of PTCH in PANC1 cells (Figure 5 B). These inhibitors apparently decrease the PTCH expression in PANC1 cells at 4 $\mu g\,mL^{-1}$. We investigated the effects of compounds **1**–**7**, **9**, **17**, and **18** on the viability of PANC1 cells by using a fluorimetric microculture cytotoxicity assay (FMCA).[27] Compounds **1**, **9**, **17**, and **18** were cytotoxic to PANC1 cells with IC_{50} values of 40, 68, 2.6, and 5.3 μM, respectively (Table 2). We also examined the cytotoxicity against a mesenchymal progenitor (C3HT10T1/2) cell line that is derived from the mouse embryonic mesoderm, which is Hh responsive but not reliant on Hh for survival. The cyctotoxicity of compounds **9**, **17**, and **18** against PANC1 cells were from three- to fivefold higher than that against C3H10T1/2 cells, although **1** showed the similar IC_{50} values of both PANC1 and C3H10T1/2 cells. On the other

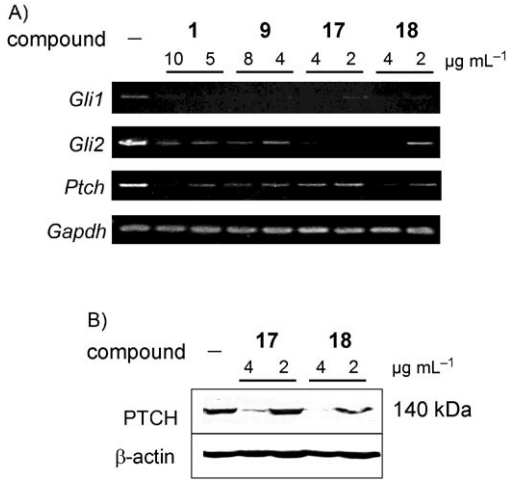


Figure 5. Effect of inhibitors on Hh/GLI signaling component expressions in PACN1 cells. A) Semiquantitative RT-PCR. PANC1 cells were incubated with or without each inhibitor, **1**, **9**, **17**, and **18** for 24 h. mRNA expression of *Gli1*, *Gli2*, and *Ptch* in untreated and inhibitor-treated cells were shown with that of *Gapdh*. B) Western blotting analysis of PTCH in PANC1 cells. PANC1 cells were treated with inhibitors **17** and **18** for 24 h, and PTCH protein expression was analyzed by Western blotting. β-Actin was used as a control.

Table 2. IC_{50} values of cytotoxicity against PANC1 and C3H10T1/2 cells.

Compound	IC_{50} values[a]	
	PANC1	C3H10T1/2
1	68.8 (15.0)	70.7 (15.4)
2	>85.4 (>20)	>85.4 (>20)
3	191 (39)	>123 (>25)
4	>227 (>50)	>113 (>25)
5	144 (32)	>112 (>25)
6	136 (34)	>100 (>25)
7	>200 (>50)	>105 (>25)
9	40.8 (12.7)	137 (42.6)
17	2.7 (1.4)	15.0 (7.9)
18	5.3 (2.7)	17.0 (8.7)
cyclopamine	8.0	24.9

[a] μM (μg mL^{-1}).

hand, cyclopamine also showed cyctotoxicities against both PANC1 and C3H10T1/2 cells with IC_{50} values of 8.0 and 24.9 μM, respectively. The cytotoxic activity of **9**, **17**, and **18** could be related to their inhibition of GLI-mediated transcription because physalins **17** and **18** showed both potent inhibition of GLI-mediated transcription and potent cytotoxicity in PANC1 cells.

Conclusions

We have identified several types of naturally occurring inhibitors of GLI-mediated transcription by using a cell-based assay; zerumbone (**1**), zerumbone epoxide (**2**), staurosporinone (**9**), 6-hydroxystaurosporinone (**10**), arcyriaflavin C (**11**), 5,6-dihydroxyarcyriaflavin A (**12**), physalin F (**17**) and physalin B (**18**). We confirmed their activities in mRNA and protein-expression experiments. These compounds have a different mechanism of action from cyclopamine, which is an inhibitor of Smo. Physalins from *Physalis minima* showed the most active GLI-mediated transcriptional inhibitory activity in our identified compounds. In addition, the compounds might be useful tools for investigating the Hh/GLI signaling pathway, and they might be good leads for the development of new agents for the treatment of cancer.

Experimental Section

Materials: Optical rotations were measured on a JASCO P-1020 polarimeter. The NMR spectra were recorded on JEOL JNM ecp600 and A500 spectrometers. High-resolution ESI-MS was obtained on a Micromas LCT Spectrometer. HPLC was carried out on Shimadzu LC-10ADvp pump that was equipped with a SPD-M10vp detector ($\lambda = 254$ nm), and a Mightysil, RP-18 GP (4.6×250 mm i.d.) for analytical HPLC and a Develosil ODS-HG-5 column (10×250 mm i.d.) for preparative HPLC, by using a H_2O/MeOH solvent system.

Cell culture conditions: HaCaT cells were cultured in Dulbecco's modified Eagle Medium (DMEM, high glucose, Wako, Osaka, Japan) that contained 5% fetal bovine serum (FBS, biowest, Miami, FL, USA) and streptomycin/penicillin (Gibco). PANC1 cells, which were provided from RIKEN BRC, were grown in RPMI-1640 medium (Wako) supplemented with 10% FBS. C3H10T1/2 cells that were provided from RIKEN BRC were maintained in DMEM (high glucose, Wako) + 10% FBS.

Plasmid construct: The pGL4-GLI BS construct was made by inserting the 12×GLI binding region of the 12GLI-TKO-luc vector, which was provided by Dr. Rune Toftgård. The GLI binding region was amplified with PCR with KOD-Plus-DNA polymerase (TOYOBO, Osaka, Japan). PCR was performed by using 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 68 °C for 30 s, and a final elongation step 68 °C for 5 min. The final 449 bp PCR product was cut with NheI and BglII, and was then inserted into NheI/BglII cut of pGL4-2.0 (Promega). The constructs were sequence verified.

Transfection: Tetracycline-regulated HaCaT cells (T-REx system) that expressed the transcriptional factor GLI1, which was provided by Drs. Frits Aberger and Gerhard Regl were transfected with pGL4-GLI BS with Lipofectamine 2000 (Invitrogen); 90% confluent HaCaT cells were plated on a 10 cm dish. On the next day, the cells were transfected with the pGL4-GLI BS luciferase construct (12 μg) together with Lipofectamine 2000 (20 μL). Two days later, puromycin (10 μg mL^{-1}) was added into the medium for stable cell line selection. By repeatedly changing the medium with puromycin, 46 transfectants were obtained, and the luciferase activity of these transfectants was checked by the addition of tetracycline to choose one for screening study.

The constructed screening system: In this constructed cell-based assay system, three plasmids (pcDNA6/TR, pcDNA3.1-GLI1, and pGL4-GLI-luc) were stably cotransfected in the assay cell. The pcDNA3.1-GLI1 plasmid produced exogenous GLI1 proteins, and pGL4-GLI-luc plasmid was a reporter construct that produced luciferase by GLI1-mediated transactivation. This assay was based on "tetracycline-regulated expression system (Tet-On system)", and the expression of GLI1 was regulated by the tetracycline repressor. The addition of tetracycline produced an amount of exogenous GLI1 proteins in the assay cell, and subsequently the luciferase expression was increased.

Natural products and plant extracts screening: With the established screening cells (HaCaT-GLI1-Luc), 94 members of our natural

products library and 192 substances from the tropical plant extract library, which was collected in the southeastern parts of Thailand were examined. In this screening, both reporter luciferase-activity-mediated GLI1 transcription and the cell viability of the treated cells were coinstantaneously determined as described in the luciferase assay. Two different final concentrations of 25 and 2.5 $\mu g\,mL^{-1}$ were performed in the natural products screening, and the plant extracts library were screened at 100 and 50 $\mu g\,mL^{-1}$. The samples that exhibited high cyctotoxicity (< 50%) at their screening concentrations were examined again at lower concentrations.

Plant material and isolation of physalins from *P. minima*: The GLI1 inhibitory screening identified *Physalis minima* as a hit plant. The whole part of *Physalis minima* was collected in Khon Kaen, Thailand in September 2001, and was identified by T.K. A voucher specimen (7-019) was maintained at the Faculty of Agriculture, Khon Kaen University. The whole parts of *P. minima* were extracted with MeOH, the MeOH extract (2.7 g) was partitioned with H_2O and *n*-hexane, EtOAc, and afforded an *n*-hexane extract (584.9 mg), an EtOAc extract (391.8 mg) and a H_2O extract (1.41 g). In the GLI1 transcriptional inhibitory assay, the hexane and EtOAc extracts were active at 10 $\mu g\,mL^{-1}$. Their luciferase reporter activities of each extract was 51% and 21%, respectively, with high cell viability (> 95%). Because TLC analysis of their extracts revealed almost the same constitutes in the *n*-hexane and EtOAc extracts, these extracts (0.9 g) were combined and were placed on silica gel column (colomn A: 30×220 mm) and were eluted with gradient mixtures of *n*-hexane/EtOAc (0:1–1:0) and EtOAc/MeOH (1:0–0:1) to give 8 fractions (fractions 1A–1H). In the GLI1 inhibitory assay of each fraction, frs. 1C (75 mg), 1D (73 mg) and 1E (18 mg) showed GLI1 inhibitory activity at 1 $\mu g\,mL^{-1}$, and the reporter luciferase activites were 26%, 17% and 36%, respectively, with > 80% viability, and frs. extracts 1F (107 mg), 1G (219 mg) and 1H (64 mg) showed activity at 10 $\mu g\,mL^{-1}$ that ranged from 40–55% of luciferase reporter activity with > 85% viability. Fraction 1D of column A was subjected to Sephadex LH-20 column chromatography (18×600 mm) with a $CHCl_3$/MeOH (1:4) mixture to give six fractions (frs. 2A–2F; fraction 2F was undissolved in a $CHCl_3$/MeOH (1:4) mixture when it was applied onto LH-20 column). Fraction 1C was also separated with the same column as fraction 1D to give five fractions (frs. 3A–3E). The GLI1-mediated transcriptional inhibitory activity was measured on each fraction, and fractions 2D, 2F, 3D were more than active at 1 $\mu g\,mL^{-1}$. Fraction 2F was one spot in the TLC analysis, and was further purified with ODS HPLC (Develosil ODS-HG-5; eluent, 50% MeOH; flow rate, 2.0 $mL\,min^{-1}$) to afford physalin F (**17**, 5.1 mg). Fraction 3D was also purified on the same ODS HPLC condition to give physalin B (**18**, 4.9 mg) and isophysalin B (**19**, 0.4 mg). Isolated physalins F and B (**17** and **18**) were examined for the GLI1-mediated transcriptional activity and cell viability at various concentrations (0.05–0.75 $\mu g\,mL^{-1}$) to give IC_{50} values.

Luciferase assay: HaCaT-GLI1-Luc cells were cultured in a 24-well plate at 2×10^5 cells in 37 °C, under 5% CO_2 atomosphere. After incubation for 12 h, 1 $\mu g\,mL^{-1}$ of tetracycline was added into each well to induce exogenous GLI1 protein expression. After induction of GLI1 protein for 12 h, the cells were washed with PBS twice, and then each concentration of samples or compounds were added. After incubation at 37 °C for 12 h, the luciferase activity was measured in a microplate luminometer (Thermo, Luminoskan Ascent, Vantaa, Finland) by using a Bright-Glo™ Luciferase Assay System (Promega) according to the manufacture's protocol. At the same time, the cyctotoxicity of the samples was measured. Briefly, the same cells (HaCaT-GLI1-Luc) were seeded at 1×10^4 cells/96-well plate at 37 °C, 5% CO_2 for 24 h. Samples at different concentrations were added at the same time as the luciferase assay, the cells were treated with the samples at 37 °C for 12 h. Cell viability was determined by fluorometric microculture cytotoxicity assay (FMCA) by using a fluoroskan ascent (Thermo).

Dual luciferase assay: 90% Confluent HaCaT cells that expressed GLI2 under tetracycline control were plated on to 24-well plates. On the next day, the cells in each well were transfected with the pGL4-GLI BS-luc reporter construct (1.0 μg) and renilla luciferase (pRL-CMV; 0.2 μg) as a transfection control by using Lipofectamine 2000 (2 μL; Invitrogen) and incubated at 37 °C. After 12 h of transfection, tetracycline (1 $\mu g\,mL^{-1}$) was added into each well to cause the induction of GLI2 protein, and then the cells were incubated at 37 °C for 12 h. The transfectant were treated with compounds **1**, **9**, **17** and **18** at 37 °C for 12 h, and then normalized luciferase activity was determined with the Dual-Glo™ Luciferase Assay system (Promega) by using a Microplate luminometer (Thermo) according to the manufacture's protocol. The experiments and individual measurements performed at least 3 times. The experiment of bcl2 promoter activity by using pGL3-bcl2 reporter plasmid was done by using the same method as mentioned above. Briefly, 90% confluent PANC1 cells were seeded on 24-well plates. After 24 h, pGL3-bcL2 reporter plasmid (1.0 μg) and pRL-CMV (0.1 μg) were transfected with Lipofectamine 2000 (2 μL) in each well. Compounds were then added 6 h after transfection. After 24 h of treatment with the compounds, the dual-luciferase assay was performed.

Cytotoxic assay against PANC1 and C3H10T1/2 cells: PANC1 and C3H10T1/2 cells, respectively, were seeded onto 96-well microtiter plates at 1×10^4 cells per well, and were preincubated for 24 h at 37 °C. The medium was replaced with fresh medium that contained different concentrations of each compound. The cells were then incubated at 37 °C for 24 h. After the medium was removed, cell proliferation was determined by fluorometric microculture cytotoxicity assay (FMCA) by using a fluorescence platereader. The ratio of the living cells was determined as the fluorescence in the sample wells was expressed as a percentage of that in the control wells, and cytotoxic activity was indicated as an IC_{50} value.

Western blotting analysis: Tetracycline (1 $\mu g\,mL^{-1}$) was added to HaCaT cells (2×10^6 cells) to express exogenous GLI1 or GLI2, while tetracycline was not added as negative control. After 12 h treatment with tetracycline, the medium was removed and different concentrations of compounds (**1**, **9**, **17**, and **18**) were added. After incubation for 24 h at 37 °C, cells were harvested, washed with ice-cold PBS, 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) that contained 1% proteasome inhibitor cocktail (Nacalai Tesgue, Kyoto, 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) that contained 5% skimmed milk for 1 h at room temperature, the blots were hybridized at room temperature for 1 h with primary antibodies. β-Acitin was used as an internal control. After washing with TBST, the blots were incubated at room temperature for 1 h with secondary antibodies that were conjugated with horseradish peroxidase. After washing, the immunocomplexes were visualized by using an ECL Advance Western detection system (GE health Care/Amersham Biosciences) and Immobilon Western (Millopore, Billerica, MA, USA). PTCH expression in PANC1 was also determined by the same method. The analysis was per-

formed by using antibodies to GLI1 (1:200), PTCH (1:200) (Santa Cruz Biotechnology, 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.

RNA isolation and semiquantitive reverse transcriptase-polymerase chain reaction (RT-PCR) analysis: Total RNA from cells that were treated with compounds **1**, **9**, **17** and **18** was isolated by using RNeasy Mini kit (Qiagen) according to the manufacture's protocol, and mRNA from the extracted total RNA was isolated by using an Oligotex-dT30 mRNA Purification Kit (TaKaRa Bio, Siga, Japan). cDNA was constructed from the mRNA by using a PrimeSTAR RT-PCR Kit (TaKaRa) according to the manufacture's instruments. Sequences for the relevant primers were as follows: Gli1, 5′-GCCGTGTAAAGCTCCAGTGAACACA-3′ and 5′-TCCCACTTTGAGAGGCCCATAGCAAG-3′ (200 bp product); Gli2, 5′-TGGCCGCTTCAGATGACAGATGTTG-3′ and 5′-CGTTAGCCGAATGTCAGCCGTGAAG-3′ (200 bp product); Ptch, 5′-TCCTCGTGTGCGCTGTCTTCCTTC-3′ and 5′-CGTCAGAAAGGCCAAAGCAACGTGA-3′ (200 bp product), and human GAPDH; 5′-ATGGGGAAGGTGAAGGTCG-3′ and 5′-TAAAAGCAGCCCTGGTGACC-3′ (70 bp product) as a internal control gene. The reaction was run for 25 to 35 cycles of 94 °C for 1 min (denaturation), 58 °C for 30 s (annealing), and 72 °C for 30 s (extension) by using TaKaRa Ex Taq Polymerase. Amplification products was subjected to electrophoresis on 3% agarose-TBE gel, stained with ethidium bromide, visualized under UV light and compared with Gapdh.

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