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Methanol Extract of *Cinnamomum cassia* Represses Cellular Proliferation and Gli-mediated Transcription in PANC-1 Human Pancreatic Cancer Cells

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Abstract – Twenty five methanolic plant extracts were investigated to determine the anticancer activity against sonic hedgehog (shh)/Gli signaling pathway dependent cancer, PANC-1 human pancreatic cancer cells, through three screening programs. All extracts were inspected their inhibitory properties on sonic hedgehog-conditioned medium (shh-CM) induced alkaline phosphatase (ALP) activity in C3H10T1/2 mouse mesenchymal stem cells to examine whether the plant extracts affect the shh/Gli signaling pathway. Next, plant extracts were screened the ability to suppress the cell proliferation of PANC-1 human pancreatic cancer cells. Finally, active plant extracts from the two screening systems were evaluated for the suppressive effect on Gli-mediated transcriptional activity in PANC-1 cells. Among active plants, *Cinnamomum cassia* suppressed Gli-mediated transcriptional activity leading to the down-regulated expression of Gli-target genes such as Gli-1 and Patched-1 (Ptch-1). This study provides the consideration for the important role of natural products in drug discovery process as well as the basis for the further analysis of active plant and potential identification of novel bioactive compounds as inhibitors of Gli and therapeutic candidates against shh/Gli signaling pathway dependent cancers.
Keywords – *Cinnamomum cassia*, Pancreatic cancer, Gli, Sonic hedgehog pathway, Alkaline phosphatase activity

Introduction

Pancreatic cancer is a highly lethal disease with an overall 5-year survival of about 5%.[1] Although many attempts have been made to improve the clinical therapies of pancreatic cancer, it needs to explore new and more effective alternatives for the treatment and prevention. It has been reported that aberrant activation of sonic hedgehog (shh)/Gli signaling pathway plays a critical role in initiation and maintenance of pancreatic cancer.[2-4] The shh/Gli signaling, which are occupied with shh ligand, 12-pass transmembrane receptor Patched (Ptch), 7-transmembrane receptor Smoothened (Smo) and transcriptional factor Gli, has been implicated not only in embryogenesis and tissue maintenance,[5] but also in growth of certain cancers with amplication of Gli or mutations in Ptch or Smo, or decontrolled gene expression.[6,7] Although the Smo inhibitors have been utilized to determine the inhibition of hedgehog signaling, the therapeutic efficacy shows limits in preclinical and clinical models. The tumors with activation of Smo-downstream components, such as a transcription factor Gli, are unaffected to Smo inhibitors.[8,9] Several studies suggested that the deregulation of Gli in mediating oncogenic shh/Gli signaling could be essential for the tumor formation.[10-13] Therefore, the inhibition of Gli in a Smo-independent mode can be a great research target to monitor shh/Gli signaling pathway activity in cell based assays.

Natural products have been used as relevant resource for anticancer properties. For instance, clinical useful anticancer agents including paclitaxel from the bark of the pacific yew tree *Taxus brevifolia* have been derived from plants.[14,15] The plant-derived anticancer agents have been discovered by variable screening programs. In addition, the screening and selection of the bioactive plant-resources have been highly important roles in the drug discovery steps. In this study, 25 methanolic plant extracts were investigated to determine the anticancer activity against shh/Gli signaling pathway dependent cancer, PANC-1 human pancreatic cancer cells. The screening process was designed with a three-tiered system, where all extracts were inspected their inhibitory properties on sonic hedgehog-conditioned medium (shh-CM) induced alkaline phosphatase (ALP) activity in C3H10T1/2 mouse mesenchymal stem cells to examine whether the plant extracts affect the shh/Gli signaling pathway. Next, plant

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extracts were screened the capability to suppress the cell proliferation of PANC-1 human pancreatic cancer cells. Finally, the extracts showing the repressive abilities on shh induced ALP activity and the proliferation of cancer cells were evaluated for the suppressive effect on Gli-mediated transcriptional activity in PANC-1 cells. The candidate plant extract was selected through these test systems, and the down-regulated mRNA levels of Gli-1 and Ptch-1 were assessed to confirm the inhibition of Gli-mediated gene expression in pancreatic cancer cells.

Experimental

Plant materials – The methanol extracts of plants were kindly provided by Professor J. H. Ryu from the College of Pharmacy, Sookmyung Women's University (SMU). The voucher specimens (No. SPH 201306S01-SPH 201306S25) were deposited in the herbarium of SMU. The herbal materials were purchased from the oriental medicinal herb market (Seoul, Korea) and the air-dried materials (50 g) were reflux extracted with methanol at 80 °C for 2 hr (× 3) to yield crude methanol extracts. All plant extracts were received as dissolved in DMSO to 100 mg/mL and stored at 4 °C until use. The final concentration of DMSO in assay system was less than 0.1%, which has no effect on cell proliferation or other cellular functions.

Cell culture and chemical reagents – PANC-1 (human pancreatic cancer cells) and C3H10T1/2 (mouse mesenchymal stem cells) cells (from ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle Medium that contained 10% FBS and streptomycin/penicillin (Gibco). GANT61 (purity: ≥ 98%), a synthetic Gli inhibitor, was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Cyclopamine (purity: ≥ 98%, Sigma-Aldrich Co.), a representative plant-derived inhibitor of Smo in the Hh pathway, was used as a positive control for the evaluation of inhibitory properties of shh/Gli signaling pathway.

Alkaline phosphatase (ALP) assay – C3H10T1/2 cells were seeding at 5×10^3 cells/well in a 96 well plate and allowed to attach for 4 hr. And then sonic hedgehog conditioned medium (shh-CM) and test materials were added. The preparation of shh-CM has been described previously.[16] After 96 hr, 0.9% NaCl with 0.2% Triton X-100 were added to the cells for 15 min to lyse. And then cell lysates were mixed with ALP substrate (4 mM p-nitrophenyl phosphate disodium) and reaction buffer (200 mM Tris-HCl pH10.5, 0.4 M 2-amino-2-methylpropanol, 8 mM $MgCl_2$), and incubated at 37 °C, in the dark, for 45 min. By using a microplate reader (Molecular Devices, CA, USA), absorbance was measured at 415 nm. The assay was performed in triplicate.

Cellular proliferation assay (MTT assay) – PANC-1 human pancreatic cancer cells were plated at a density of 3,000 cells/well in a 96 well plate. Cells were treated with various concentrations of plant extracts for 3 days. And then 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, 5 mg/mL) was added for 4 hr and lysed with DMSO. Absorbance at 570 nm was measured by using a microplate reader (Molecular Devices, CA, USA). The assay was performed in triplicate.

Gli-dependent luciferase reporter assay – The assay was performed as previously described.[16] Briefly, the plasmids of Gli-dependent firefly luciferase reporter construct (gift from Prof. G.U. Bae at Sookmyung Women's University, Korea) and β-galactosidase reporter were transiently transfected to PANC-1 cells. These cells were treated with various concentrations of plant extracts. After incubation for 20 hr at 37 °C, cellular firefly luciferase and β-galactosidase activities were measured by using chemiluminescence.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis – PANC-1 cells (2.5×10^5 cells/60 mm dish) were treated for 20 hr with or without test samples. After washing twice with phosphate buffered saline (PBS), total RNA was isolated from the cell pellet using a RNA-isolation Trizol reagent (Life Technologies, Paisley, UK). Two micrograms of RNA was reverse transcribed into cDNA using reverse transcriptase and random hexamer. The PCR samples, contained in the reaction mixture, comprised mixture buffer, dNTP, Taq DNA polymerase (Promega, Madison, WI) and primers (sense and antisense). The sense and antisense primers for Gli-1, Ptch-1 and GAPDH were 5'-GCCGTGTAAAGC TCCAGTGAACACA-3' and 5'-TCCCACTTTGAGAGG CCCATAGCAAG-3'; 5'-TCCTCGTGTGCGCTGTCTTC CTTC-3' and 5'-CGTCAGAAAGGCCAAAGCAACGTG A-3'; 5'-TGTGATGGGTGTGAACCACGAG-3' and 5'-TG CTGTTGAAGTCGCAGGAGAC-3', respectively. The amplified DNA was separated on 1.5% agarose gels and stained with ethidium bromide.

Statistical analysis – Data were presented as means ± S.D. for the indicated number of independently performed experiments. Statistical analysis was assessed by one-way analysis of variance (ANOVA) and Student's *t*-test. A p value of < 0.05 was considered to be statistically significant difference.

Result and Discussion

Shh/Gli signaling pathway comprises many factors

although the essential pathway has few components. Shh ligand binds and inactivates the receptor Ptch. Of the two homologs of Ptch, Ptch-1 and Ptch-2, the role of Ptch-1 is better understood.[17] Without ligand stimulation, Ptch-1 normally inhibits the activation of Smo. Upon presence of the ligand, the Smo is relieved from Ptch-1 inhibition and send a signal to the ultimate effectors of the pathway, the transcription factors Gli family (Gli-1, Gli-2, and Gli-3), leading to cell growth. Gli-1 and Gli-2, the prime transcriptional effectors, have been known to be involved in tumor formation. In addition, the importance of Gli-1 in tumor development and progression is well documented in human cell culture systems.[18-21]

The preliminary determination of the effect of plant extracts on inhibition of shh/Gli signaling pathway was made by assessment of the shh-CM induced ALP activity in C3H10T1/2 mouse mesenchymal stem cells. The shh/Gli signaling pathway has been known to be involved in the osteoblast differentiation, which results in increased ALP activity, a marker of osteoblast differentiation.[22] Therefore, the inhibition of shh/Gli signaling pathway induces the weakened ALP activity. Twenty five plant extracts were examined the effects on the shh-CM induced ALP activity in C3H10T1/2 cells. Cyclopamine, a Smo inhibitor, was used as a positive control. As shown in Table 1, fourteen kinds of plant extracts showed 50% or greater inhibition at 10 μg/mL, in which no cytotoxicity was exhibited. In addition, the extracts of *Asiasarum heteroptropoides*, *Cinnamomum cassia*, *Citrus unshiu*, *Dictamnus dasycarpus*, *Elsholtzia ciliata*, *Glycyrrhiza uralensis*, *Inula japonica*, *Phellodendron amurense*, *Poncirus trifoliata*, *Sophora subprostrata*, *Vitex rotundifolia*, and *Zizyphus jujuba* demonstrated the potent inhibitory effects of ≥ 70%. These results suggest that 14 plant extracts affect shh-CM induced osteoblast differentiation although the exact steps of pathway are not considered. Moreover, the repressed ALP activity by inhibition of Smo-downstream can be expected as one of the various occasions. The active plants from this first screening system could be the candidates for the inhibition of shh/Gli signaling pathway.

However, other assay systems are needed to select the active materials that inhibit cancer cell growth by inhibition of terminal effector Gli, Smo-downdstream of shh/Gli signaling pathway. Measurement of the proliferation of Gli positive pancreatic cancer cells can be used as a next screening system. We investigated whether the 25 kinds of plant extracts could affect the cancer cell proliferation. The extracts were treated to PANC-1 human pancreatic cancer cells at various concentrations for 72 hr. GANT61, a Gli inhibitor, was used as a positive control because PANC-1 cells had been known as the constitutive activation of Gli and highly proliferative cancer cells. [21] About all plant extracts showed the inhibitory cellular proliferation in dose dependent manner. However, only five plant extracts (*Cinnamomum cassia*, *Clematis mandshurica*, *Inula japonica*, *Sophora subprostrata* and *Vitex rotundifolia*) displayed ≥ 50% of inhibitory effects on pancreatic cellular proliferation at 50 μg/mL (Table 2). In addition, these active extracts demonstrated the potent inhibitory

Table 1. The inhibitory effects of plant extracts on shh-CM induced ALP activity in C3H10T1/2 mouse mesenchymal stem cells

Botanical names of plants (Family)	Parts[a]	Inhibition (%)[b]
Angelica tenuissima (Umbelliferae)	Rh	55.4 (± 1.2)*
Asiasarum heteroptropoides (Aristolochiaceae)	R/Rh	71.3 (± 2.6)*
Bupleurum falcatum (Umbelliferae)	R	46.7 (± 2.1)*
Chaenomeles sinensis (Rosaceae)	F	27.5 (± 1.6)*
Chrysanthemum indicum (Compositae)	FL	17.2 (± 2.3)*
Cinnamomum cassia (Lauraceae)	B	70.7 (± 3.5)*
Citrus unshiu (Rutaceae)	IP	82.0 (± 2.9)*
Clematis mandshurica (Ranunculaceae)	R	46.1 (± 3.1)*
Dictamnus dasycarpus (Rutaceae)	Rh	99.1 (± 1.1)*
Elsholtzia ciliata (Labiatae)	S/L	78.2 (± 4.1)*
Gentiana macrophylla (Gentianaceae)	R	17.3 (± 2.9)*
Glycyrrhiza uralensis (Leguminosae)	R	78.8 (± 6.2)*
Inula japonica (Compositae)	FL	99.2 (± 0.8)*
Kalopanax pictus (Araliaceae)	B	49.4 (± 4.1)*
Kochia scoparia (Chenopodiaceae)	F	50.4 (± 3.7)*
Lonicera japonica (Caprifoliaceae)	F	26.7 (± 3.1)*
Lonicera japonica (Caprifoliaceae)	S/L	42.8 (± 2.3)*
Ostericum koreanum (Umbelliferae)	R	28.0 (± 3.2)*
Phellodendron amurense (Rutaceae)	B	93.0 (± 0.8)*
Poncirus trifoliata (Rutaceae)	F (immature)	99.0 (± 0.2)*
Schizonepeta tenuifolia (Labiatae)	Sp	26.4 (± 6.1)*
Scutellaria baicalensis (Labiatae)	R	26.0 (± 5.2)*
Sophora subprostrata (Leguminosae)	R	80.1 (± 2.9)*
Vitex rotundifolia (Verbenaceae)	F	99.8 (± 0.1)*
Zizyphus jujuba (Rhamnaceae)	F	70.0 (± 4.8)*
Cyclopamine		96.3[c] (± 0.6)*

[a]B: bark, F: fruit, FL: flower, IP: immature peel, L: leaves, R: root, Rh: rhizome, S: stems, Sp: spike
[b]at concentration of 10 μg/mL; [c] at concentration of 5 μM; * $p < 0.05$ versus vehicle

Table 2. The suppressive effects of plant extracts on cell proliferation of PANC-1 pancreatic cancer cells

Botanical names of plants	Inhibition (%)		
	10 μg/mL	20 μg/mL	50 μg/mL
Angelica tenuissima	< 10	16.9 (± 2.1) *	19.2 (± 3.5) *
Asiasarum heteroptropoides	< 10	< 10	< 10
Bupleurum falcatum	19.0 (± 2.6) *	21.2 (± 1.9) *	31.4 (± 2.2) *
Chaenomeles sinensis	< 10	< 10	< 10
Chrysanthemum indicum	< 10	< 10	19.7 (± 4.1) *
Cinnamomum cassia	14.7 (± 3.5) *	46.9 (± 4.7) *	50.0 (± 1.3) *
Citrus unshiu	< 10	23.2 (± 3.1) *	47.0 (± 4.2) *
Clematis mandshurica	42.4 (± 3.6) *	51.9 (± 1.1) *	66.8 (± 2.3) *
Dictamnus dasycarpus	22.7 (± 3.6) *	27.6 (± 3.2) *	30.3 (± 3.1) *
Elsholtzia ciliata	< 10	10.9 (± 4.2)	26.3 (± 2.4) *
Gentiana macrophylla	< 10	< 10	< 10
Glycyrrhiza uralensis	< 10	< 10	< 10
Inula japonica	55.0 (± 5.0) *	64.0 (± 3.8) *	85.7 (± 1.5) *
Kalopanax pictus	< 10	< 10	< 10
Kochia scoparia	< 10	< 10	41.3 (± 3.7) *
Lonicera japonica	< 10	< 10	10.9 (± 1.0)
Lonicera japonica	31.5 (± 2.2) *	32.4 (± 1.4) *	33.0 (± 2.9) *
Ostericum koreanum	< 10	< 10	< 10
Phellodendron amurense	< 10	< 10	25.9 (± 1.7) *
Poncirus trifoliata	12.7 (± 0.9) *	22.0 (± 1.6) *	31.9 (± 2.4) *
Schizonepeta tenuifolia	< 10	< 10	13.8 (± 1.1) *
Scutellaria baicalensis	< 10	< 10	< 10
Sophora subprostrata	16.6 (± 2.0) *	38.6 (± 1.9) *	51.5 (± 4.1) *
Vitex rotundifolia	61.7 (± 2.1) *	66.2 (± 1.4) *	78.7 (± 1.1) *
Zizyphus jujuba	< 10	< 10	< 10
GANT61		82.2[d] (± 0.8) *	

[d] at concentration of 30 μM; * $p < 0.05$ versus vehicle

effects (≥ 70% at 10 μg/mL) on shh induced ALP activity except that *Clematis mandshurica* showed 46.1% of inhibitory activity at 10 μg/mL (Table 1 and 2). These results indicate that the candidates for the inhibitors of Smo-downstream could be *Cinnamomum cassia*, *Inula japonica*, *Sophora subprostrata* and *Vitex rotundifolia* which exhibit the potent inhibitory properties on shh induced ALP activity as well as on the pancreatic cancer cell proliferation.

PANC-1 human pancreatic cancer cells have been reported that transcription factor Gli is highly activated, which leads to the stimulated cellular proliferation and to the overexpression of Gli-target genes such as Gli-1 and Ptch-1.[21] Furthermore, mutant KRAS activates Gli-1 and Gli activity may be modulated by altering the activity of pathway such as RAF/MEK/MAPK pathway that interact with shh signaling in pancreatic cancer.[23,24] Therefore, third screening way was performed to test whether these active four plant extracts could suppress the Gli-mediated transcriptional activity. Mutation or overexpression of Gli, downstream of Smo, has been shown to activate the shh/Gli signaling where Smo inhibitors are ineffective.[7,25] Thus, measurement of Gli transcriptional activity, the final event in the pathway, would be reasonable strategy for screening the inhibitors of Gli-mediated transcription. Four active plant extracts, *Cinnamomum cassia*, *Inula japonica*, *Sophora subprostrata* and *Vitex rotundifolia*, were treated to PANC-1 cells transfected with Gli-firefly luciferase reporter and β-galactosidase reporter. After 20 hr, the cellular firefly luciferase and β-galactosidase activities were measured. GANT 61, a synthetic Gli inhibitor as a positive control, showed the 59.0% of inhibitory effect at 30 μM. As shown in Table 3, *Inula japonica*, *Sophora subprostrata* and *Vitex rotundifolia* showed the ≤ 40% of suppressive Gli-transcriptional activities. However, these three plant extracts strongly

Table 3. The inhibitory effects of plant extracts on Gli-dependent transcriptional activity in PANC-1 cells

Botanical names of plants	Inhibition (%)		
	10 μg/mL	20 μg/mL	50 μg/mL
Cinnamomum cassia	8.3 (± 1.2) *	34.6 (± 2.1) *	77.3 (± 1.3) *
Inula japonica	< 10	19.2 (± 1.0) *	38.6 (± 4.2) *
Sophora subprostrata	< 10	< 10	< 10
Vitex rotundifolia	20.6 (± 3.1) *	26.6 (± 2.6) *	36.5 (± 2.4) *
GANT61	–	–	59.0[d] (± 1.2) *

[d] at concentration of 30 μM; * $p < 0.05$ versus vehicle

suppressed the pancreatic cancer cell proliferation and shh induced ALP activity (Table 1 and 2). This gives us an assumption that these inhibitory actions of three plant extracts might be involved in indirectly altered activities of shh/Gli signaling pathway by modulation of other pathway such as RAF/MEK/MAPK pathway. The extract of *Cinnamomum cassia* repressed Gli-mediated transcriptional activity dose dependently and demonstrated the 77.3% potent inhibitory effect at 50 μg/mL (Table 3). These results suggests, at least in part, that the inhibitory properties of *Cinnamomum cassia* on the differentiation of C3H10T1/2 cells to osteoblasts and on the cancer cell proliferation are involved in the inhibition of Gli-mediated transcriptional activity.

Cinnamomum cassia contains several active components such as cinnamic aldehyde, cinnamyl aldehyde, salicylaldehyde, anhydrocinneylanol, cinnasassiols and cinnamtannins.[26,27] They have been found to possess anti-oxidant, anti-microbical, anti-inflammation, anti-diabetic and anti-tumor effects.[28-32] To further identify the repressive Gli-mediated transcription by extract of *Cinnamomum cassia*, the expressions of Gli-target genes such as Gli-1 and Ptch-1 were examined by RT-PCR analysis. The mRNA levels of Gli-1 and Ptch-1 were detected by treatment of extract of *Cinnamomum cassia* to PANC-1 cells where the expressions of Gli-1 and Ptch-1 were highly increased by over-activated transcription factor Gli. GAPDH was used as an internal control. As shown in Fig. 1, the mRNA levels of Gli-1 and Ptch-1 were decreased in dose dependent manner at treatment group of *Cinnamomum cassia*, whereas vehicle treatment showed highly over-expressed Gli-1 and Ptch-1 mRNA. This observation suggests that the Gli-1 and Ptch-1 mRNA expressions are down-regulated through the suppression of Gli-mediated transcriptional activity by treatment of *Cinnamomum cassia*.

In conclusion, this study demonstrated the successful stream of the screening procedure of bioactive plants with anti-cancer properties in shh/Gli-dependent cancers. First, 25 plants extracts were screened for their ability to inhibit

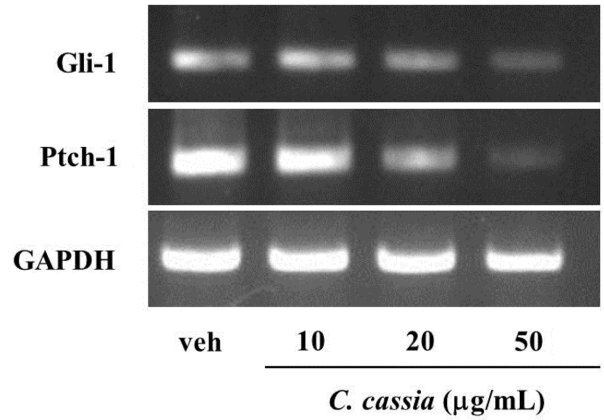

Fig. 1. Effect of *Cinnamomum cassia* extract on the mRNA expressions of Gli-target genes in PANC-1 cells. Cells were treated with various concentrations of *Cinnamomum cassia* extract for 20 hr. The mRNA levels for Gli-1, Ptch-1 and GAPDH were determined by RT-PCR from total RNA extracts. GAPDH was used as an internal control. Images are the representative of three independent experiments that shows similar results.

the shh induced differentiation of C3H10T1/2 cells to osteoblasts in an ALP assay. And then the extracts were examined the effects on proliferation of pancreatic cancer cells. The results obtained from these two screening systems eliminated the poor candidates on the basis of down-regulated shh/Gli-dependent proliferative properties. Selected four active plants were further screened to affect the Gli-mediated transcriptional activity in PANC-1 cells. Among four active plants, *Cinnamomum cassia* suppressed Gli-mediated transcriptional activity leading to the down-regulation of Gli-1 and Ptch-1 expression. This work provides the basis for the further analysis of this active plant and potential identification of novel bioactive compounds as inhibitors of Gli and therapeutic candidates against shh/Gli signaling pathway dependent cancers.

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