

3',6-Dimethoxy-3'',4''-Methylenedioxy-2,5-Epoxy lignan-4'-ol Inhibited Glioma-Associated Oncogene

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Abstract

Aberrant activation of Hedgehog (Hh) signaling pathway has been linked to the development of cancers. A naturally occurring Hedgehog inhibitor, 3',6-dimethoxy-3'',4''-(methylenedioxy)-2,5-epoxy lignan-4'-ol (DMEO), isolated from *Piper nigrum*, exhibited selective cytotoxicity against human pancreatic (PANC1) with no toxic effect on normal cells. This compound blocked the translocation of GLI transcription factors into the nucleus in PANC1. RNA interferences of the Smoothed (Smo) function in PANC1 treated with the compound downregulated the mRNA expression of Ptch.

Keywords: Hedgehog signaling; GLI; Smo; PANC1; MRNA expression; Ptch

Introduction

The Hh signaling pathway has become an important regulator of tissue patterning, stem cell maintenance and cancer growth [1]. In cancer cells, binding of the Hh protein to the membrane receptor patched 1 (PTCH) releases its inhibitory effect on Smo. Activated Smo further transduces downstream cascades to activate the glioma-associated oncogene (GLI) family of transcription factors [2]. Recent papers reported that GLI signaling pathway has been considered as a promising target for selective cancer therapy.

In some types of human cancers, Hh/GLI signaling is activated because of mutations in Ptch or Smo, leading to cancer formation and progression. Searching for bioactive natural products targeting Hh/GLI signaling thus may promote an effective anticancer-strategy. We identified several Hh/GLI-mediated transcriptional inhibitors from plants [3-6] including 3',6-dimethoxy-3'',4''-(methylenedioxy)-2,5-epoxy lignan-4'-ol (DMEO) which was isolated from *Piper nigrum* [7]. We have previously adapted the concept of nano magnetic beads to the fast isolation of GLI inhibitory compounds, using GLI-GST culture which was immobilized on the beads and plant extracts [8]. DMEO was bound to GLI-magnetic bead thus may be a lead constituent for the inhibition of Hh/GLI-dependant cancer.

GLI families, which are activated by the aberrant Hh/GLI signaling in the cells, contribute to the progression of pancreatic [9] and prostate [10] cancer cells. Most known Hh inhibitors repress the pathway by inhibiting Smo thus inactivate Ptch function; however, one of the oncogenic Smo mutants (SmoM2) is apparently resistant to cyclopamine [11] and most Smo inhibitors did not reduce the proliferation levels of medullablastoma and cancer related to downstream lesions [12].

In the present study, to provide a novel bioactive agent that could inhibit GLI, the cytotoxicity of DMEO and its underlying mechanism were elucidated in cellular extracts of PANC-1. To verify the function of Smo in PANC1 during the Hh inhibition of DMEO, RNA interference work was also performed.

Materials and Methods

Compound

DMEO was isolated from *Piper nigrum* according to the *target oriented-isolation* method [7]. Compound DMEO was isolated as a white

amorphous solid and gave the molecular formula $C_{21}H_{24}O_6$, as deduced from HR-FABMS m/z 395.1294 (calcd for $C_{21}H_{24}O_6Na$, 395.1305). The IR absorption bands suggested the presence of hydroxyl (3490 cm^{-1}) (br) and aromatic ($1620, 1450\text{ cm}^{-1}$) groups and methylenedioxy (935 cm^{-1}). The UV absorption maxima were at UV (MeOH) λ_{max} 282 nm ($\log \epsilon$ 3.1) and 230 nm ($\log \epsilon$ 3.0). For identification of the compound, ^1H NMR spectra were recorded at 400 MHz on an AC-250 Bruker NMR spectrometer, and elemental analyses were carried out to get analytical results compared to literatures.

Target-oriented isolation using gli-bound magnetic beads

GST-GLI was bound to magnetic beads according to the manual procedure (Invitrogen). MeOH extract of *Piper nigrum* fruits in EtOH solution (370 mg in EtOH, 50 mL) was added to GLI-bound magnetic beads (2 mL). The mixture was gently kept for 2h at -4°C prior to centrifugation for 10 min (12,000 rpm, -4°C). The supernatant solution was removed and the pellet (15.5 mg) was subjected to preparative HPLC [Capcell Pack C.18 type Acr, $4.6 \times 250\text{ mm}$; MeOH : H_2O (3 : 2); flow rate: 2.0 mL/min; RI and UV detection at 254 nm] to obtain DMEO (8.2 mg).

Cytotoxicity test

PANC1, C3H10T1/2 (RIKEN BRC) or DU145 (Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University) were seeded in black 96-well plates at 1×10^4 cells per well. After incubation at 37°C for 24h, medium was replaced by DMEO at different concentrations (1.9 μM ; 3.8 μM ; 7.6 μM and 15.2 μM). Cell proliferation was determined by the fluorometric microculture cytotoxicity assay (FMCA), and IC_{50} values were determined. Assays were performed in triplicate.

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Received October 25, 2014; Accepted November 27, 2014; Published December 04, 2014

Citation: Rifai Y, Arai M, Ishibashi M (2014) 3',6-Dimethoxy-3'',4''-Methylenedioxy-2,5-Epoxy lignan-4'-ol Inhibited Glioma-Associated Oncogene. J Biomol Res Ther 3: 117. doi: [10.4172/2167-7956.1000117](http://dx.doi.org/10.4172/2167-7956.1000117)

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Isolation of cellular extracts

PANC1 cells were seeded into 10 cm dishes (2×10^6 cells) and incubated for 24h at 37°C. Compounds DMEO at various concentrations were added. After 24h incubation, cells were washed with PBS and then collected by scraping the whole parts. Protein lysate was prepared with lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 10 mM EDTA, 1 mM sodium orthovanadate, and 0.1 mM NaF) containing a 1% proteasome inhibitor cocktail (Nacalai Tesque, Japan), and then centrifuged at 13000 rpm, 4°C for 30 min.

Isolation of cytosolic and nuclear proteins

PANC1 cells were seeded into 60 mm dishes (2×10^6 cells), and incubated for 24h at 37°C, after which cells were harvested with trypsin and centrifuged at 1000 rpm at 4°C for 5 min. Protein lysates were prepared in the same way as the isolation of the whole cellular extract. Nuclear and cytosolic extracts were prepared using NE-PER nuclear and cytosolic extraction reagents (Pierce) according to the manufacturer's instructions.

Western blotting

Protein lysates of the whole extract, cytosol or nucleus were blotted using standard procedures. To detect GLI1 (150 kDa), PTCH (140 kDa) and BCL-2 (26 kDa), anti-GLI1 (Santa Cruz Biotechnology), anti PTCH (Santa Cruz Biotechnology), and anti BCL-2 (Sigma) were used as specific primary antibodies followed by anti-goat IgG (Sigma), anti-rabbit IgG, and anti-mouse IgG (Amersham Biosciences) as the second antibodies. β -actin (45 kDa) was used as an internal control.

RNA interference experiments

PANC1 cells were seeded into 6 cm dishes (4×10^5 cells) and incubated for 12h. RPMI-1640 medium (containing 10 % FBS) was removed and compound DMEO at concentrations of 4.0 μ M and 8.0 μ M were added. After 12h incubation, cells were transfected with small interfering RNA (20 nM) (SMO siRNAs, Santa Cruz Biotechnology) using SiPORT™ NeoFX™ (Ambion) and OPTI-MEM® (Invitrogen), as described by the manufacturer. Non-targeting siRNA at the same concentration served as a control (DMEO was replaced by DMSO) was transfected. To confirm the transfection efficiency of RNA interference,

Western blotting was performed prior to quantitative RT-PCR. First and second antibodies (anti Smo, Santa Cruz Biotechnology and anti-rabbit IgG, Jackson Immuno Research Lab. Inc.) were used then the band was detected using enhanced chemiluminescence light (GE Healthcare Biosciences).

Real-Time RT-PCR

Total RNA was extracted using an Rneasy Mini kit (Qiagen), and cDNA was synthesized using the RT-PCR SuperScript III Platinum Two Step qRT-PCR Kit (Invitrogen). The mRNA levels of Ptch were measured on a Mx3000P QPCR system (Stratagene) at 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). Each amplification reaction was performed in triplicate. mRNA quantification is expressed in arbitrary units and was normalized to an internal control GAPDH.

Results and Discussion

GLI inhibitory activity of DMEO (IC_{50} value of 4.1 μ M) was examined by using the luciferase assay whereas the cytotoxicity of DMEO against pancreatic cancer cells (PANC1), prostate cancer cells (DU145) and normal cells (C3H10T1/2) was checked using the FMCA system. The results revealed that the new epoxy lignan compound was cytotoxic against PANC1 cells (IC_{50} value of 3.6 μ M) and DU145 cells (IC_{50} value of 3.4 μ M) but did not affect normal cell lines (Figure 1a and 1b). Cytotoxicities may contribute to the inhibition of GLI transcriptional activity.

To understand the molecular mechanism underlying the Hh signaling inhibitory effect of DMEO, we checked the expression of a GLI-related gene (Ptch) using real-time quantitative RT-PCR (Figure 2a). Compared with the control group, mRNA expression of PANC1 treated with DMEO was slightly down regulated at a concentration of 4.0 μ M. The compound DMEO might be able to modulate Ptch expression in PANC1 due to the inhibition of GLI transcriptional activity.

To verify the function of Smo in PANC1 during the Hh inhibition of DMEO, we knocked down Smo expression in PANC1 cells by siRNA and performed real-time quantitative RT-PCR on its mRNA

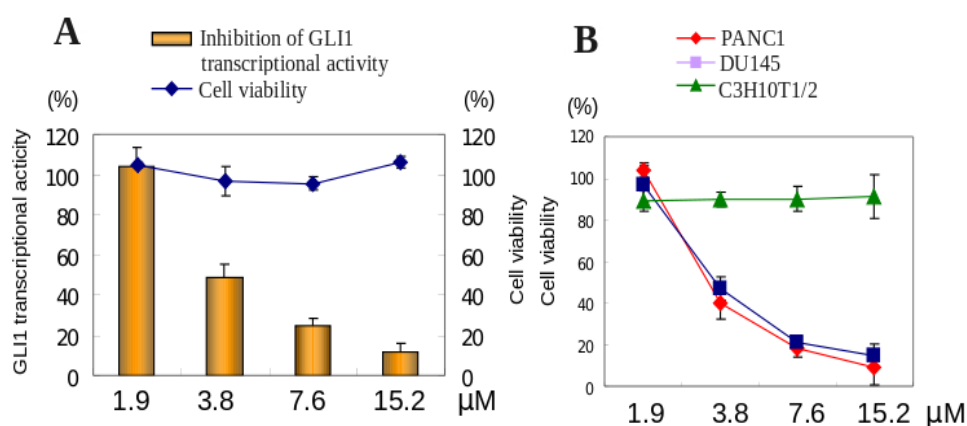
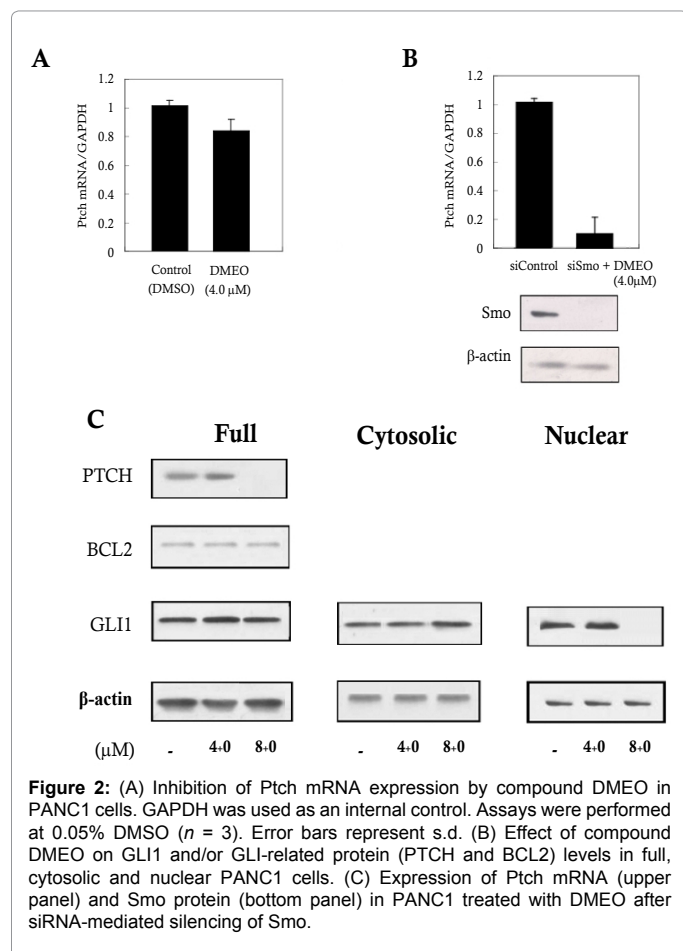


Figure 1: (A) Inhibition of GLI1-mediated transcriptional activity (solid columns) and cell viability (solid curves) of DMEO. HaCaT-GLI1-Luc cells were seeded onto a 96-well plate (2×10^5 cells per well) and then treated with compounds 12h after tetracycline addition. Cell viability and luciferase activity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



expression. Western blot results confirmed that there was a proved total depletion of the Smo protein level after the silencing process (Figure 2b). Accordingly, silencing of Smo siRNA significantly reduced the expression of Ptch mRNA expression in PANC1 cells treated with DMEO (Figure 2c). These results thus indicated that DMEO is a potential GLI inhibitor in the nuclei site of PANC-1 without Smo interference.

The effect of DMEO on downstream events was identified by checking the protein levels of full, cytoplasmic and nuclear PANC1 after treatment with DMEO. Our Western blot result showed that

treatment with DMEO at a concentration of 4.0 μM led to a significant decrease in the protein level of nuclear GLI1 in PANC1. The compound also reduced the expression of a GLI-related gene Ptch but not BCL-2 in the protein level of full cytoplasmic PANC1 according to a dose-dependent manner (Figure 2c). This result was in agreement with the previous report stated that Ptch was a repressive Hh receptor. Thus elevated expression of Ptch results in the concomitant expression of Hh target genes, including GLI.

Acknowledgement

This work was supported by Ministry of Science and Technology of Indonesia through grant of SiNas 2013. We are grateful to Dr. Sasaki from RIKEN (Japan) for the kind provision of cultured GST and to Prof. Elly Wahyudin the dean of Faculty of Pharmacy from Hasanuddin University for providing necessary research facilities.

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