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Inhibition of Phospholipase Cγ1 and Cancer Cell Proliferation by Lignans and Flavans from *Machilus thunbergii*

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Thirteen compounds were isolated from the CH_2Cl_2 fraction of *Machilus thunbergii* as phospholipase Cγ1 (PLCγ1) inhibitors. These compounds were identified as nine lignans, two neolignans, and two flavans by spectroscopic analysis. Of these, 5,7-di-*O*-methyl-3',4'-methylenated (-)-epicatechin (**12**) and 5,7,3'-tri-*O*-methyl (-)-epicatechin (**13**) have not been reported previously in this plant. In addition, seven compounds, machilin A (**1**), (-)-sesamin (**3**), machilin G (**5**), (+)-galbacin (**9**), licarin A (**10**), (-)-acuminatin (**11**) and compound **12** showed dose-dependent potent inhibitory activities against PLCγ1 *in vitro* with IC_{50} values ranging from 8.8 to 26.0 μM. These lignans, neolignans, and flavans are presented as a new class of PLCγ1 inhibitors. The brief study of the structure activity relationship of these compounds suggested that the benzene ring with the methylene dioxy group is responsible for the expression of inhibitory activities against PLCγ1. Moreover, it is suggested that inhibition of PLCγ1 may be an important mechanism for an antiproliferative effect on the human cancer cells. Therefore, these inhibitors may be utilized as cancer chemotherapeutic and chemopreventive agents.

Key words: *Machilus thunbergii*, Lauraceae, Phospholipase Cγ1, Lignan, Flavan, Methylene dioxy group, Antiproliferation of human cancer cells

INTRODUCTION

Aberrations in cell signaling pathways result in hyperproliferative diseases, and interventions of these pathways have served as useful targets for cancer chemotherapy and chemoprevention. Among the enzymes involved in the signaling pathways, phosphatidylinositol specific phospholipase C (PLC) is a key enzyme. It generates two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP_2) and plays a pivotal role in transmembrane signal transduction. Of its isozymes, PLCγ is essential for proliferation by mitogens (Berridge, 1993; Nishizuka, 1992; Rhee and Bae, 1997). There are a number of reports that this enzyme is related to abnormal cell proliferation including cancer and its inhibitors could be used as lead compounds in anticancer agents (Hill *et al.*, 1994; Lee and Kim, 2001; Powis, 1993) Our groups have reported a biflavonoid (Lee *et al.*, 1996b), an isocoumarin (Lee *et al.*, 1999a), long chain phenols (Lee *et al.*, 1998), norlignans (Lee *et al.*, 1996a), a retrochalcone (Park *et al.*, 1998), and triterpene esters (Lee *et al.*, 1999b, 2000) as new classes of PLCγ1 inhibitors from medicinal plants.

Machilus thunbergii Sieb. et Zucc. (Lauraceae) has been used to treat dyspepsia, abdominal pain, and distention in Korean traditional medicine (Kim, 1984). Various lignans have been reported as hepatoprotective components of this plant (Yu *et al.*, 2000). The CH_2Cl_2 extracts of this plant showed inhibitory activity against PLCγ1, but the compounds responsible for this activity have not been previously reported. Therefore, this paper deals with the isolation of active components from *M. thunbergii*, PLCγ1 activity relationships, and antiproliferative effects in human cancer cell lines.

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MATERIALS AND METHODS

General

IR spectra were obtained with a Perkin Elmer 1710 spectrophotometer. NMR spectra were taken on a JEOL LA 300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) spectrometer. EIMS spectra were obtained using a VG Trio-2 spectrometer. TLC was carried out on silica gel 60 F_{254} and RP-18 F_{254} plates (Merck, Darmstadt, Germany). Column chromatography was performed over silica gel 60 (Merck, particle size 230-400 mesh or 15 μm) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden).

Plant materials

The bark of *Machilus thunbergii* Sieb. et Zucc. (Lauraceae) was purchased from the Korean Export and Import Federation of Drugs in Seoul, Korea and identified by Dr. Dae Suk Han, an emeritus professor at the College of Pharmacy at Seoul National University. A voucher specimen (SNUPH-0052) has been deposited in the College of Pharmacy herbarium at Seoul National University in Korea.

Extraction and isolation

The dried bark (5 kg) of *M. thunbergii* was extracted three times using 80% MeOH in an ultrasonic apparatus for 3 h. This extract was evaporated *in vacuo* to yield a 80% MeOH extract (500 g), which was subsequently fractionated using CH_2Cl_2 and H_2O. Separation of inhibitory principles from the CH_2Cl_2 extract (40.3 g) was carried out as follows: It was fractionated by column (12×48 cm) chromatography over a silica gel (3.6 kg, 230-400 mesh) using a *n*-hexane-EtOAc (20:1) → EtOAc-MeOH (1:1) solvent system, to give 8 fractions (1-8). Column chromatography of fractions 1-3 over a silica gel (800 g, 4.2 × 30 cm, 230-400 mesh, *n*-hexane-EtOAc=20:1) yielded 8 subfractions (9-16). Compounds **5** and **10** (158.6 mg) were separated from fraction 9 by vacuum column chromatography (60 g, 2.2×25 cm, 15 μm, *n*-hexane-EtOAc=100:1) and purified by recrystallization with *n*-hexane. Fraction 12 was further chromatographed using a silica gel (140 g, 2×42 cm, 230-400 mesh, *n*-hexane-EtOAc=10:1) and Sephadex LH-20 (1×33 cm, MeOH). Final purification was achieved by either recrystallization to yield **3** (1.65 g, *n*-hexane), **13** (15 mg, *n*-hexane-EtOAc=5:1), and **11** (13 mg, *n*-hexane-EtOAc=20:1) or semipreparative HPLC (Microsorb® C_{18}, 254 nm, 2 mL/min) on RP-18 eluted with H_2O-CH_3CN-MeOH (W-A-M) to yield **1** (12 mg, t_R 16.55 min, W-A-M=37:13:50), **7** (18 mg, t_R 13.44 min, W-A-M=37:13:50), **6** (34 mg, t_R 20.03 min, W-A-M=18:22:60), and **2** (19 mg, t_R 12.78 min, W-A-M=18:22:60). Compounds **4**, **8**, **9**, and **12** were separated and purified from fraction 13 by the same procedure that applied to fraction 12. Compounds **4** and **12** were further purified by recrystallization, whereas **8** and **9** were purified by semipreparative RP HPLC with retention times of 15.82 min (**8**, 13 mg, W-A-M=20:0:80) and 22.79 min (**9**, 10 mg, W-A-M=30:20:50).

Enzyme and chemicals

PLCγ1 from bovine cerebellum was purified to homogeneity (over 95% purity) through DE-52, matrix green gel affinity, phenyl 5-PW, and MONO O column chromatography (Rhee *et al.*, 1991). L-3-phosphatidyl inositol (PI), (*N*-[2-hydroxyethyl]piperazine-*N*'-[4-butanesulfonic acid]) (HEPES), ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) and sodium deoxycholate (SDC) were purchased from Sigma Chemical Co. (St. Louis, MO, and USA). [^{3}H-inositol] PI and a cocktail solution were obtained from Amersham (UK, England). Amentoflavone, which was isolated previously by our group (Lee *et al.*, 1996b), was used as a positive control in the PLCγ1 assay.

In vitro PLCγ1 assay

The PLCγ1 assay was performed by the methodology of Rhee *et al.* (1991). The enzyme solution was dissolved in 50 mM HEPES/NaOH (pH 7.0), 3 mM $CaCl_2$, and 1 mM EGTA. The reaction mixture contained 0.02 μCi [^{3}H-*inositol*] PI, 10 nM PI, and 0.1%-SDC. The enzyme reaction was initiated by adding the enzyme at 37 °C and was terminated after 10 min by adding 1 mL of $CHCl_3$-MeOH-conc. HCl (50: 50: 0.3, by volume) and 0.3 mL of 1 N HCl containing 3 mM EGTA. The mixture was vortexed and centrifuged at 2000 rpm for 10 min. The aqueous layer was placed in a scintillation vial and the radioactivity of [^{3}H-inositol] IP was counted. Each inhibitor was tested in triplicate at each of eight points. Amentoflavone was used as a positive control.

Cell lines and culture media

Cell lines (A-549, MCF-7, and HCT-15) were obtained from the Korean Cell Line Bank at Seoul National University in Seoul, Korea. The cell culture media (RPMI-1640) and an antibiotic (penicillin-streptomycin) were purchased from Gibco-BRL (Grand Island, NY, USA). Fetal bovine serum was obtained from Hyclon (Logan, UT, USA).

Growth inhibition assay of cancer cell

A549 (human lung carcinoma), MCF-7 (human breast adenocarcinoma), and HCT-15 (human colon adenocarcinoma) were grown in a RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum plus 1% antibiotics. Cells were cultured in T-flasks in a CO_2 incubator supplied with 5% CO_2 and 95% humid air at 37 °C. Cells were maintained at 5~10×10^4 cells/mL with a

subculture 2-3 times a week. 0.05% Trypsin-EDTA was used for dissociating monolayer cells. Cell suspension ($1\sim8\times10^4$ cells/well) of the log phase was added to each well of a 96-well plate to determine the human cancer cell growth inhibition of compounds, and incubated in a CO_2 incubator at 37°C. After one day, the compounds were treated and the cells were cultured for additional 2 days. The final DMSO concentration was adjusted to 0.5% in all samples. Each experiment was performed in triplicate. Viable cells were counted by the SRB method (Skehan *et al.*, 1990). The cell growth inhibition index was determined as IC_{50}. The drug concentration resulting in 50% growth inhibition was compared to the untreated control. Adriamycin was used as a positive control.

RESULTS AND DISCUSSION

The CH_2Cl_2 extract of the bark of *M. thunbergii* showed inhibitory activity of PLCγ1 (79.5%) at 100 μg/mL concentration. Thirteen compounds were isolated from this extract by sequential column chromatography over silica gel, sephadex LH-20 and reverse-phase semipreparative HPLC. The structures of these compounds were determined by comparison of spectroscopic data with authentic samples and literature values as nine lignans **1-9**, two neolignans **10-11** and two flavans **12-13**, namely, machilin A (**1**), *meso*-dihydroguaiaretic acid (**2**), (-)-sesamin (**3**), (-)-isoguaiacin (**4**), machilin G (**5**), nectandrin A (**6**) and B (**7**), (+)-galbelgin (**8**), (+)-galbacin (**9**), licarin A (**10**), (-)-acuminatin (**11**), 5,7-dimethyl-3',4'-methylene (-)-epicatechin (**12**), and 5,7,3'-tri-*O*-methyl (-)-epicatechin (**13**) (Fig. 1). Among these, **12** and **13** have not been reported previously from this plant (Achenbach *et al.*, 1987; Ajaneyulu *et al.*, 1977; El-Feraly *et al.*, 1982; Holloway and Scheinmann, 1974; King and Wilson, 1964; Miyamura *et al.*, 1983; Shimomura *et al.*, 1987, 1988).

Compounds **1-13** were evaluated for their abilities to inhibit PLCγ1 *in vitro* (Table I). Of these, four lignans **1, 3, 5, 9**, two neolignans **10**, **11**, and one flavan **12** exhibited dose dependent inhibitory activities on PLCγ1 with the IC_{50} of 8.8, 9.8, 18.5, 14.0, 15.8, 26.0, and 10.1 μM for **1**, **3**, **5**, **9**, **10**, **11**, and **12**, respectively (Table I). When the activities of lignans were compared, it was found that the ones having a methylene dioxy group exhibited more potent activity than those not having it. In the case of flavans, it was found that the methylene dioxy group is also meaningful for inhibitory activity against this enzyme. IC_{50} of **12** was 10.1 μM, whereas IC_{50} of **13** was greater than 125 μM. In other words, these results suggested that the methylene dioxy functionality is important for inhibition of the PLCγ1.

Two neolignans, licarin A (**10**) and (-)-acuminatin (**11**) showed the inhibitory activities with the IC_{50} values of 15.8

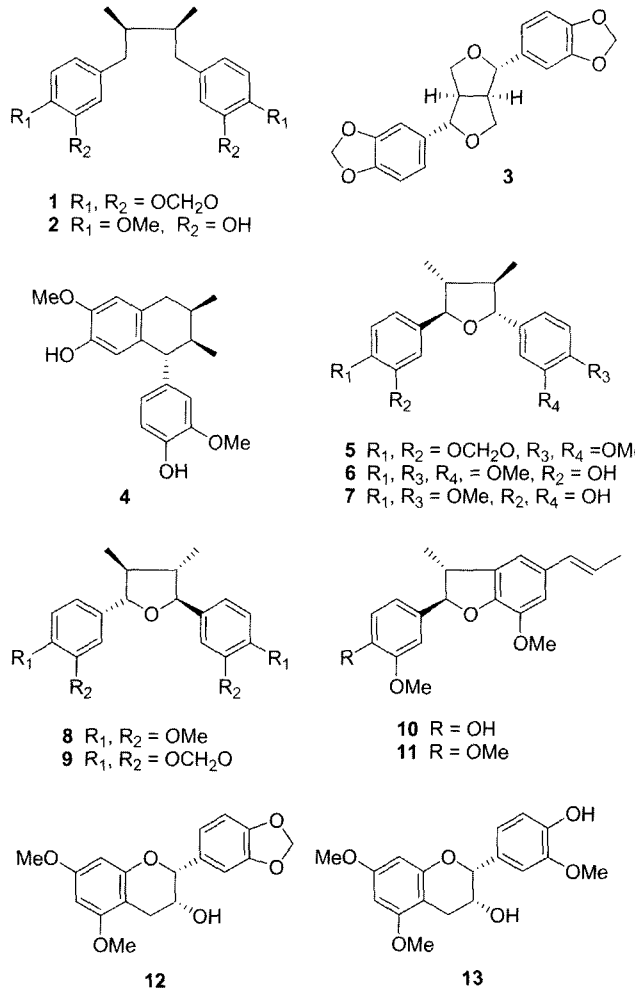

Fig. 1. Chemical structures of compounds **1-13** from *M. thunbergii*

Table I. Inhibitory effects of compounds **1-13** on PLCγ1 *in vitro*

Compounds	IC_{50} value[a] (μM)
1	8.8±1.1
2	>125 (141.2)[b]
3	9.8±0.9
4	>125
5	18.5±1.3
6	>125
7	>125
8	>125
9	14.0±1.5
10	15.8±1.3
11	26.0±1.5
12	10.1±0.8
13	>125
Amentoflavone	29.0±1.5

[a] Data were expressed as mean ± SE of three experiments
[b] Compound **2** showed IC_{50} value of 141.2 μm

Table II. Antiproliferative effects of compounds **1-13** on human cancer cell lines

Compound	IC_{50} value (μM)		
	A-549	MCF7	HCT-15
1	12.4	12.4	7.9
2	>50	>50	>50
3	4.4	3.4	11.0
4	>50	>50	16.9
5	1.4	2.7	8.3
6	>50	>50	>50
7	40.3	11.3	>50
8	13.5	37.8	>50
9	6.2	7.9	7.9
10	2.0	1.6	10.0
11	3.9	2.8	8.0
12	0.7	3.3	9.8
13	>50	>50	>50
Adriamycin	0.7	13.4	0.01

and 26.0 μM, respectively. This fact indicated that aromatic OH might be necessary for the activity of neolignans.

Several reports have suggested that PLCγ1 plays a key role in proliferation and progression of human cancer (Arteaga *et al.*, 1991; Hill *et al.*, 1994; Lee *et al.*, 2001; Noh *et al.*, 1994; Powis, 1993). Accordingly, antiproliferative activities of these compounds were also tested on three human cancer cell lines, HCT-15 (colon), MCF-7 (breast), and A549 (lung). The compounds that inhibited PLCγ1 (**1**, **3**, **5**, **9**, **10**, **11**, and **12**) (Table II) showed strong antiproliferative activities (IC_{50} 0.7-11.0 μM), whereas compounds **2**, **4**, **6**, and **13**, which did not show this enzyme activity, were also very weakly active on these cancer cells. However, compounds **7** and **8** were moderate active on A549 and MCF7 (IC_{50} 11.3-40.3 μM). These results suggest that inhibition of PLCγ1 may be an important mechanism for antiproliferative effect on the human cancer cells. Therefore, these PLCγ1 inhibitors may be worthy candidates as cancer chemopreventive and chemotherapeutic agents.

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