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Dibenzocyclooctadiene Lignans From *Schisandra chinensis* Protect Primary Cultures of Rat Cortical Cells From Glutamate-Induced Toxicity

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A methanolic extract of dried Schisandra fruit (*Schisandra chinensis* Baill.; Schisandraceae) significantly attenuated the neurotoxicity induced by L-glutamate in primary cultures of rat cortical cells. Five dibenzocyclooctadiene lignans (deoxyschisandrin, gomisin N, gomisin A, schisandrin, and wuweizisu C) were isolated from the methanolic extract; their protective effects against glutamate-induced neurotoxicity were then evaluated. Among the five lignans, deoxyschisandrin, gomisin N, and wuweizisu C significantly attenuated glutamate-induced neurotoxicity as measured by 1) an inhibition in the increase of intracellular [Ca^{2+}]; 2) an improvement in the glutathione defense system, the level of glutathione, and the activity of glutathione peroxidase; and 3) an inhibition in the formation of cellular peroxide. These results suggest that dibenzocyclooctadiene lignans from *Schisandra chinensis* may possess therapeutic potential against oxidative neuronal damage induced by excitotoxin.

Key words: dibenzocyclooctadiene lignans; excitotoxic cell damage; glutathione defense system; neuroprotective activity; *Schisandra chinensis*

L-glutamate is a major amino acid associated with central excitatory neurotransmission as occurs in neuronal survival, synaptogenesis, neuronal plasticity, learning, and memory processes in the brain (Albright et al., 2000). However, glutamate also causes neuronal cell loss (Choi, 1988; Coyle and Puttfarcken, 1993) by two distinct forms of response, acute and delayed (Choi, 1985, 1987). Glutamate-induced neuronal cell death may be involved in neurological disorders such as seizures (Lipton and Rosenberg, 1994), ischemia, and spinal cord trauma (Heintz and Zoghbi, 2000) and neurodegenerative disorders such as Alzheimer's disease (Michaelis, 2003) and Parkinson's disease (Mattson et al., 1999). Thus, neuroprotection against glutamate-induced neurotoxicity has been a therapeutic strategy for preventing and/or treating both acute and chronic forms of neurodegeneration (Muir and Lees, 1995; Trist, 2000).

In Oriental countries, natural products have been used for the treatment of certain neurological illnesses. Therefore, we have examined several natural products for an ability to protect rat cortical cell cultures from the deleterious effects of the neurotoxicant glutamate as a primary screening method. In the course of screening extracts of natural products, we found that the total extract of ripe Schisandra fruits (*Schisandra chinensis* Baill.; Schisandraceae) could protect neuronal cells against glutamate-induced neurotoxicity. In Oriental societies, ripe Schisandra fruits have been used as a tonic (Liu, 1985). The fruit of *S. chinensis* was known to be enriched in lignans; more than 30 lignans have been isolated from this fruit (Nakajima et al., 1983). Several lignans, including wuweizisu C and gomisin A, have been reported to protect the liver from hepatotoxicity induced by carbon tetrachloride, galactosamine, and acetaminophen (Hikino et al., 1984; Kiso et al., 1985; Yamada et al., 1993; Ko et al., 1995). Pharmacological studies of lignans have also revealed antiinflammatory (Wang et al., 1994), anticancer (Yasukawa et al., 1992), and anti-HIV effects (Fujihashi et al., 1995). To date, however, there are no reports concerning their specific neuroprotective activity. Therefore, we attempted to isolate the neuroprotective constituents of the methan-

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olic extract of the fruits. In the present study, we isolated five lignans from the fruits and identified them as deoxyschisandrin (=schisandrin A), gomisin A, gomisin N (=schisandrin B), schisandrin, and wuweizisu C (=schisandrin C). Among the five lignans, deoxyschisandrin, gomisin N, and wuweizisu C exhibited significant neuroprotective activities against glutamate-induced neurotoxicity in primary cultures of rat cortical cells. Therefore, we tried to elucidate the neuroprotective mechanism of the three lignans in vitro using neuronal cultures injured by glutamate.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and Hank's balanced salt solution (HBSS) were obtained from Gibco (Grand Island, NY). All other chemicals were purchased from Sigma (St. Louis, MO).

Isolation of Lignans

Dried fruits of *S. chinensis* (20 kg) were extracted for 3 hr with 80% MeOH (three times, each with 10 liters) by using a reflux apparatus that yielded an extract (1.5 kg) upon removal of the solvent in vacuo. The MeOH extract was then suspended in distilled water and partitioned with *n*-hexane. The *n*-hexane fraction (50 g) was fractionated by extensive column chromatography over silica gel by using an *n*-hexane:EtOAc gradient, which yielded eight fractions. After silica gel column chromatography of fraction 2, five compounds were isolated separately and subsequently identified as deoxyschisandrin, gomisin N, gomisin A, schisandrin, and wuweizisu C by comparison with spectroscopic data reported previously (Ikeya et al., 1980).

Cortical Cell Culture

Primary cultures of mixed cortical cells containing both neurons and glia were prepared from ~17–19-day-old fetal rats (Sprague-Dawley) as described previously (Jang et al., 2002). In brief, embryonic cortical tissues were collected in cold Leibovitz-15 medium and incubated for 30 min in Ca^{2+}-Mg^{+}-free HBSS containing 0.25% trypsin. The digested tissue was triturated 10 times with a fine-bore Pasteur pipette. For most routine biochemical assays, the cell suspension was plated on 15-mm dishes (Falcon Primaria; Becton Dickinson) coated with collagen at a density of 5×10^5 cells/dish. To assay NO content and antioxidative enzyme activity, we used a density of 3×10^6 cells/dish on 60-mm dishes (Falcon Primaria). The cortical cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 10 IU/ml penicillin, and 10 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air-5% CO_2. After 3 days in culture, cell division of nonneuronal cells was halted by adding 5-fluoro-2′-deoxyuridine (50 μM). Cultures were allowed to mature for at least 2 weeks before being used for experiments. Our mixed cortical cultures consisted of approximately ~70–75% cells immunopositive for neuron-specific enolase and ~25–30% cells immunopositive for glial fibrillary acidic protein (Jang et al., 2002).

Assessment of Neuronal Cell Viability

The dibenzocyclooctadiene lignans tested were dissolved in dimethylsulfoxide (final concentration in culture 0.1%). Seventeen-day-old cortical cell cultures were used in assessing the isolated lignans' ability to protect neurons from excitotoxin-induced oxidative damage. The cultures were then exposed to 50 μM glutamate and maintained for 24 hr. After the incubation, the cultures were assessed for the extent of neuronal damage ("treatment throughout"). In some experiments, the cultures were treated with the lignans for 1 hr before exposure ("pretreatment") or after exposure ("posttreatment") to 100 μM L-glutamate for 30 min. After an additional 24 hr of incubation in DMEM in the absence (pretreatment) or presence (posttreatment) of the lignans, the cultures were assessed for viability by MTT assay, which reflects mitochondrial succinate dehydrogenase function (Mosmann, 1983).

In some experiments, cultures were pretreated with the appropriate lignans for 1 hr before exposure to 50 μM *N*-methyl-D-aspartate (NMDA) in HEPES-buffered salt solution containing 15 mM glucose and 10 μM glycine (pH 7.4) for 30 min or to 50 μM kainic acid (KA) and 10 μM MK-801 for 3 hr (Kim et al., 2002). The cultures were then washed and maintained in DMEM for 24 hr in the absence of lignans. Data are expressed as the percentage protection relative to vehicle-treated control cultures: 100 × [optical density (OD) of excitotoxin- + lignan-treated cultures − OD of excitotoxin-treated cultures]/(OD of control cultures − OD of glutamate-treated cultures).

Measurement of Nitrite and Calcium Content

The level of nitric oxide (NO) was determined by measuring the content of nitrite released into the medium by using the method of Dawson et al. (1994). The culture medium was reacted with Greiss reagent, and the absorbance was read at 550 nm. The concentration was determined against a nitrite standard curve. The content of intracellular calcium ($[Ca^{2+}]_i$) was determined with the fluorescent dye Fura 2-AM by ratio fluorometry (Kim et al., 2002). In brief, 1 hr before exposure to 50 μM NMDA/10 μM glycine or 50 μM KA/10 μM MK-801, cultures grown on glass cover slides were treated with lignans and 5 μM Fura 2-AM in phosphate-buffered saline (pH 7.2) at 37°C in a humidified atmosphere of 95% air-5% CO_2. The change of $[Ca^{2+}]_i$ was measured 10 min after exposure to NMDA or KA with a spectrofluorometer by exciting cells at 340 and 380 nm and measuring light emission at 520 nm. Calcium concentration was calculated according to the method of Grynkiewicz et al. (1985).

Measurement of Cellular Peroxide

The relative levels of free radicals, i.e., peroxide, in cultured cells were measured with the oxidation-sensitive compound 2,7-dichlorofluorescein diacetate (DCF-DA) by the method of Goodman and Mattson (1994). Cells were loaded with DCF-DA (50 μM, 50 min incubation), followed by three washes in HBSS. DCF fluorescence was then determined by exciting cells with light at 485 nm and measuring emitted light at 530 nm.

Measurement of Glutathione Peroxidase Activity and Glutathione Content

Cells from four culture plates were pooled in 2 ml of 0.1 M phosphate buffer (pH 7.4) and homogenized. The homogenate was centrifuged for 30 min at 3,000*g* at 4°C. The supernatant, consisting of the cytosolic + mitochondrial fractions, was used for assay. The activity of glutathione peroxidase was measured by the method of Flohe and Gunzler (1984). Total glutathione (GSH + GSSG) levels in the supernatant were determined spectrophotometrically by using the enzymatic cycling method of Tietz (1969). Protein content was measured by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

Statistical Analysis

The evaluation of statistical significance was determined by one-way ANOVA and, if significant, group means were compared by post hoc analysis by using Tukey's multiple comparison of means.

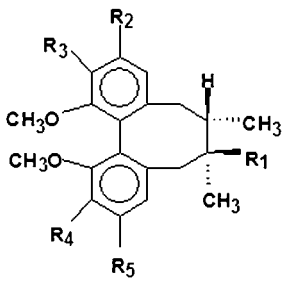
RESULTS

S. chinensis Protected Cortical Neurons From Glutamate-Induced Neurotoxicity

During our search for natural products that would be neuroprotective against glutamate-induced toxicity in primary rat cortical cultures, we discovered that the methanolic extract of dried Schisandra fruits (*S. chinensis*) showed significant protective activity (50.2% protection from glutamate-induced neurotoxicity at 100 μg/ml; $P < .01$). Therefore, we attempted to isolate neuroprotective compounds from the extracts by using bioactivity-guided isolation techniques. Five lignans were isolated from the extract and identified as deoxyschisandrin (=schisandrin A), gomisin A, gomisin N (=schisandrin B), schisandrin, and wuweizisu C (=schisandrin C; Fig. 1). The neuroprotective activity of these five lignans was initially evaluated by assessing the viability of cultured cortical neurons injured by L-glutamate in the treatment throughout paradigm. Under phase-contrast microscopy, in cortical cultures exposed to 50 μM glutamate for 24 hr, approximately 60% of neurons died in the pattern typical of necrosis, which is manifested by swelling of the cell body and neuritic fragmentation (Fig. 2). The viability of nonneuronal cells was not affected. Among the five lignans, wuweizisu C, gomisin N, and deoxyschisandrin attenuated glutamate-induced neurotoxicity (Fig. 2) to a significant dose-response "plateau" at concentrations ranging from 100 nM to 5 μM as measured by MTT assays (Fig. 3).

Dibenzocyclooctadiene Lignans Protected Cortical Neurons From Glutamate-Induced Neurotoxicity in a Different Manner

To investigate the mechanism of action of wuweizisu C, gomisin N, and deoxyschisandrin, the neuroprotective activity of these lignans against glutamate-induced toxicity was initially evaluated by a timed exposure to the lignans before (pretreatment; see Table I) or after (post-



Compounds	R_1	R_2	R_3	R_4	R_5
Deoxyschisandrin	H	OCH_3	OCH_3	OCH_3	OCH_3
Gomisin A	OH	-OCH_2O-		OCH_3	OCH_3
Gomisin N	H	-OCH_2O-		OCH_3	OCH_3
Schisandrin	OH	OCH_3	OCH_3	OCH_3	OCH_3
Wuweizisu C	H	-OCH_2O-		-OCH_2O-	

Fig. 1. Chemical structures of the five lignans isolated from *Schisandra chinensis*.

treatment; see Fig. 4) excitotoxic challenge. Although these lignans showed equipotent neuroprotective activity in a pretreatment paradigm, they showed somewhat different protective effects in a posttreatment scenario. Pretreatment with deoxyschisandrin significantly attenuated glutamate-induced neurotoxicity more than posttreatment with the lignan. By contrast, pretreatment with wuweizisu C was less effective than posttreatment with this lignan. Interestingly, wuweizisu C showed significant neuroprotective activity when administered 3 hr after glutamate exposure at concentrations ranging from 100 nM to 5 μM. Gomisin N showed less selectivity in its neuroprotective activity in either pre- or posttreatment scenarios.

Glutamate exerts its effect by acting on both NMDA and non-NMDA receptors (Krieglstein, 1997). To reveal whether wuweizisu C, gomisin N, and deoxyschisandrin act on glutamate receptors, two excitotoxins, NMDA and KA, were used to induce selective receptor-mediated neurotoxicity in primary cultures of rat cortical cells (Table II). Wuweizisu C had more effective neuroprotective activity against KA-induced toxicity than against NMDA-induced toxicity. Gomisin N, like wuweizisu C, also produced selectivity against KA-induced neurotoxicity but was found to be slightly less effective. However, deoxyschisandrin did not show significant neuroprotectivity against NMDA- or KA-induced toxicity.

Wuweizisu C and Gomisin N Inhibited Ca^{2+} Influx Induced by KA and NO Overproduction Induced by Glutamate

Treatment of primary cultured rat cortical cells with glutamate induces glutamatergic receptor-mediated neu-

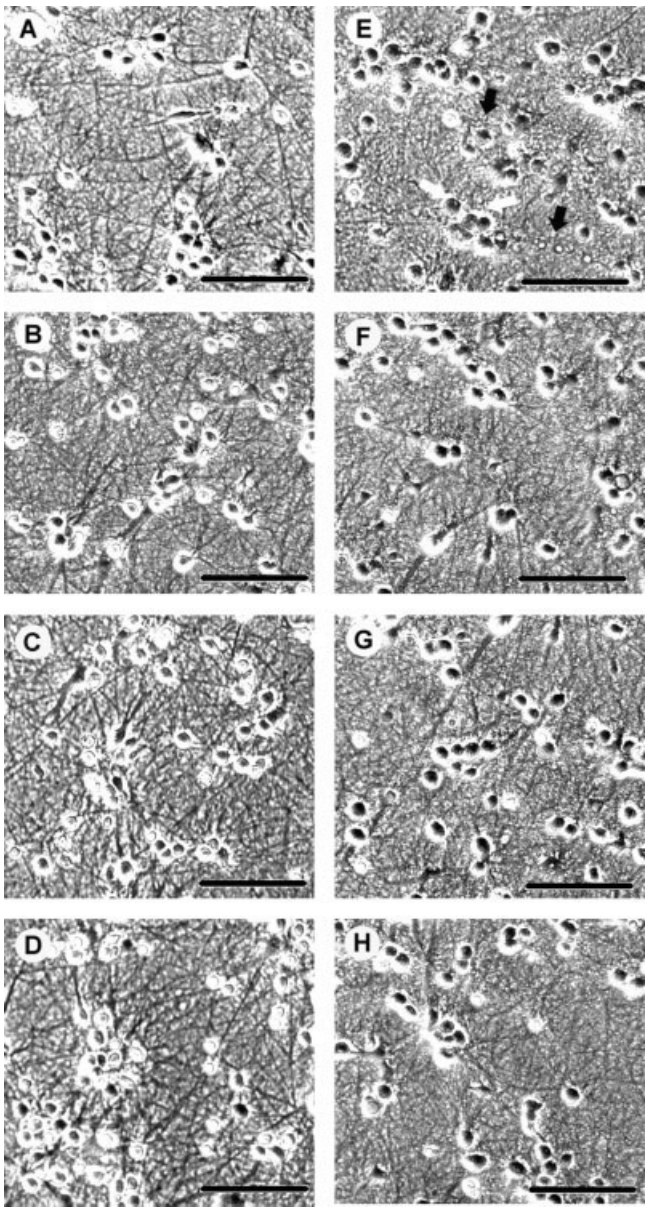


Fig. 2. Dibenzocyclooctadiene lignans of *Schisandra chinensis* protect primary cultures of rat cortical neurons from glutamate-induced neurotoxicity. Phase-contrast photomicrographs of control neurons (**A**) and neurons in the presence of 5 μM deoxyschisandrin (**B**), 5 μM gomisin N (**C**), or 5 μM wuweizisu C (**D**) without glutamate as well as neurons exposed to 50 μM glutamate in the absence (**E**) or presence of 5 μM deoxyschisandrin (**F**), 5 μM gomisin N (**G**), or 5 μM wuweizisu C (**H**). Arrows point to markedly swollen cell bodies and neurite fragmentation, respectively. Scale bars = 100 μm.

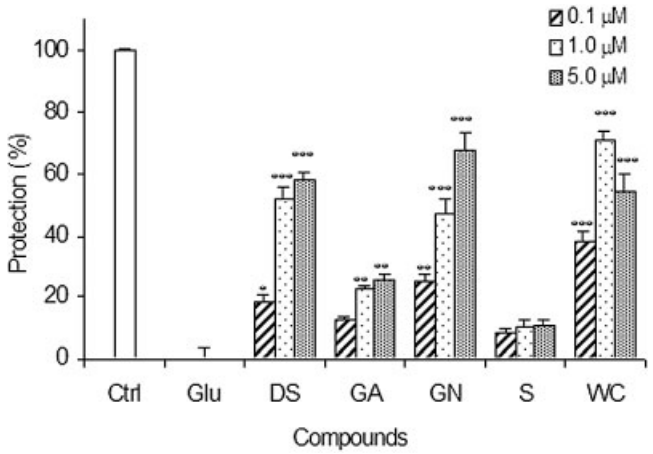


Fig. 3. Effects of dibenzocyclooctadiene lignans from *Schisandra chinensis* on primary cultures of rat cortical cells damaged by glutamate in the treatment throughout paradigm. Rat cortical cultures were treated with deoxyschisandrin (DS), gomisin A (GA), gomisin N (GN), schisandrin (S), or wuweizisu C (WC) for 1 hr before exposure to 50 μM glutamate and then maintained for 24 hr in the presence of the lignans. Cell viability was measured by the MTT assay. The control (Ctrl) was not treated with glutamate. ODs of the Ctrl and glutamate-challenged (Glu) were 1.19 ± 0.11 and 0.73 ± 0.04, respectively. The values shown are the mean ± SEM of three experiments (five or six cultures per experiment). Glutamate-injured cells differ significantly from control at $P < .001$. ⋆$P < .05$, ⋆⋆$P < .01$, ⋆⋆⋆$P < .001$ vs. glutamate-intoxicated cells (ANOVA and Tukey's test).

TABLE I. Neuroprotective Activity of Deoxyschisandrin, Gomisin N, and Wuweizisu C on Glutamate-Induced Neurotoxicity in a Pretreatment Paradigm†

	Protection (%)		
	0.1 μM	1.0 μM	5.0 μM
Control		100.0 ± 1.9	
Glutamate-injured[a]		0.0 ± 0.6	
Deoxyschisandrin	17.5 ± 2.3⋆	24.1 ± 3.1⋆	43.0 ± 3.1⋆⋆⋆
Gomisin N	24.8 ± 1.9⋆⋆	45.5 ± 1.2⋆⋆⋆	48.0 ± 1.9⋆⋆⋆
Wuweizisu C	29.5 ± 2.3⋆⋆	59.6 ± 2.1⋆⋆⋆	54.2 ± 2.8⋆⋆⋆

†Cell viability was measured by MTT assays. The control (Ctrl) was not treated with glutamate. Optical densities (ODs) of the Ctrl and glutamate-challenged (Glu) were 1.18 ± 0.09 and 0.75 ± 0.07, respectively. The values shown are the mean ± SEM of three experiments (five or six cultures per experiment). Pretreatment: Treatment with lignan was conducted 1 hr prior to the glutamate insult. Asterisks indicate significance vs. glutamate-intoxicated cells (ANOVA and Tukey's test).

[a]Glutamate-injured cells differ significantly from the control at a level of $P < .001$.

⋆$P < .05$.

⋆⋆$P < .01$.

⋆⋆⋆$P < .001$.

rotoxicity characterized by acute influx of excess calcium (Choi, 1988). Thus, we determined the effect of these lignans on the incremental change in $[Ca^{2+}]_i$ by excess NMDA or KA (Fig. 5). As shown in Figure 5, among the three lignans, only deoxyschisandrin significantly, but not effectively, blocked the increase of $[Ca^{2+}]_i$ induced by excess NMDA. However, Ca^{2+} influx provoked by excess KA was significantly blocked by treatment with wuweizisu C and gomisin N. When wuweizisu C or gomisin N was used as a pretreatment at a concentration of 1 μM,

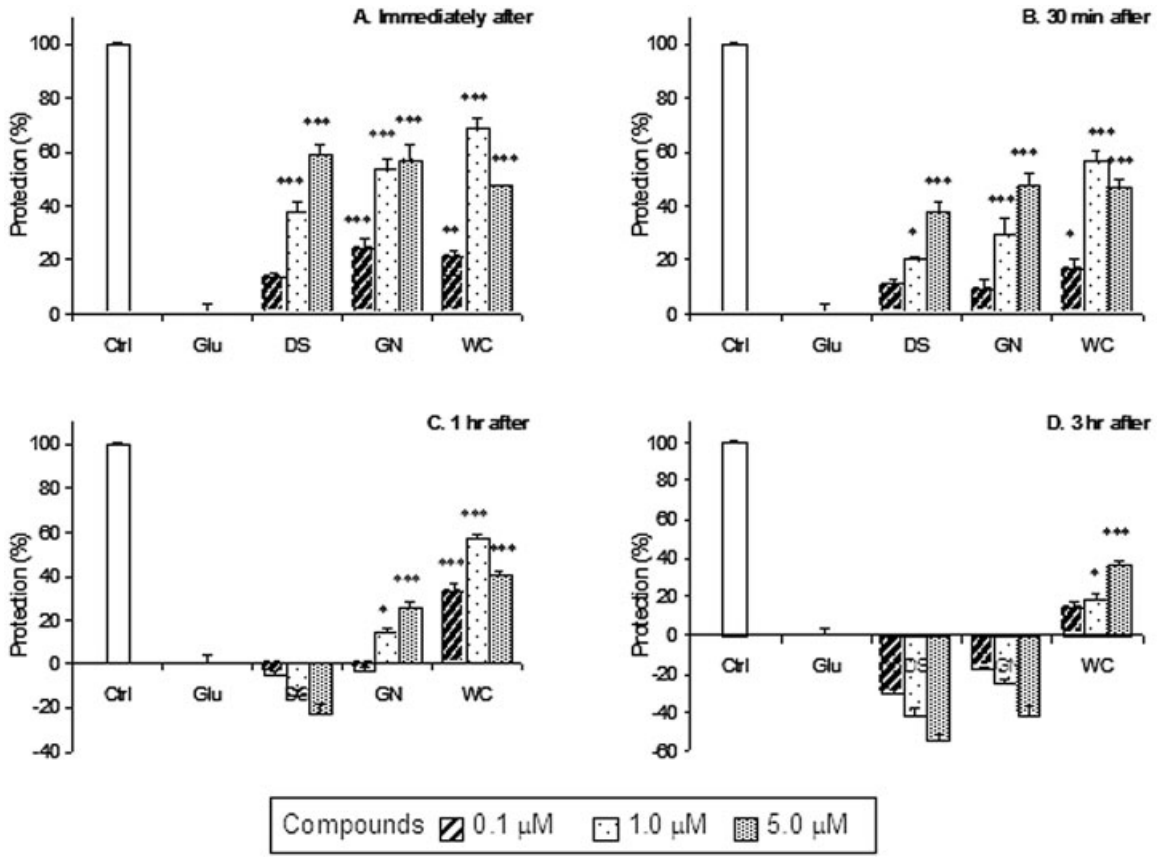


Fig. 4. Effects of deoxyschisandrin, gomisin N, and wuweisizu C on glutamate-damaged primary cultures of rat cortical cells in a posttreatment paradigm. Cortical cultures were exposed to 100 μM glutamate for 30 min, washed, and maintained in DMEM for 24 hr in the presence of compound for the posttreatment paradigm. Cell viability was measured by the MTT assay. The Ctrl was not treated with glutamate. ODs of the Ctrl and Glu were 1.16 ± 0.05 and 0.72 ± 0.06, respectively. The values shown are the mean ± SEM of three experiments (five or six cultures per experiment).Glutamate-injured cells differ significantly from the control at $P < .001$. $^{\star}P < .05$, $^{\star\star}P < .01$, $^{\star\star\star}P < .001$ vs. glutamate-intoxicated cells (ANOVA and Tukey's test).

the $[Ca^{2+}]_i$ was 50.6% or 55.7% of that in KA-treated cells, respectively (control: 75.0 ± 8.1; KA-injured: 346.0 ± 8.7; wuweizisu C-treated: 212 ± 6.3 nM; gomisin N-treated: 225.9 ± 6.3 nM).

A significant change in Ca^{2+} influx elicited by a brief exposure to glutamate or NMDA causes the overactivation of Ca^{2+}-dependent enzymes, such as nitric oxide synthase (NOS). Eventually, the overproduction of NO by NOS activation is involved in glutamate neurotoxicity through the increased formation of reactive oxygen species induced by excess glutamate (McDonald and Johnston, 1990). Therefore, we examined the effects of wuweizisu C, gomisin N, and deoxyschisandrin on the level of NO in glutamate-injured cultures. NO production in those cortical cultures damaged by glutamate increased about 4.4 times compared with normal cultures (Table III). In spite of the failure to inhibit Ca^{2+} influx induced by NMDA by treatment with all three lignans, our results showed that wuweizisu C, gomisin N, and deoxyschisandrin significantly reduced NO production (Table III).

Dibenzocyclooctadiene Lignans Reduced Cellular Peroxide by Improving the Antioxidant Defense System

The mammalian brain has defensive mechanisms against oxidative stress such as glutathione (GSH), glutathione peroxidase (GSH-px), catalase, glutathione disulfide reductase, and superoxide dismutase (Spina et al., 1992; Sampath et al., 1994). Gomisin N and wuweizisu C were previously reported to enhance glutathione antioxidative systems such as mitochondrial GSH status and GSH-px activity in several experimental designs of oxidative damage (Ip et al., 1997, 2000; Yim and Ko, 1999). This being the case, we tried to determine whether wuweizisu C, gomisin N, and deoxyschisandrin affected the glutathione antioxidative defense system in our cortical cultures. In our cortical culture system, wuweizisu C, gomisin N, and deoxyschisandrin attenuated the decrease in GSH-px activity and GSH content in cultures damaged by glutamate (Table IV). However, their effects on the

TABLE II. Neuroprotective Activity of Deoxyschisandrin, Gomisin N, and Wuweisizu C on NMDA or KA-Injured Rat Cortical Cells†

	Concentration (μM)	Protection (%)	
		NMDA-injured[a]	KA-injured[a]
Deoxyschisandrin	0.1	5.1 ± 2.7	11.1 ± 4.3
	1.0	20.8 ± 2.8★	11.7 ± 2.5
	5.0	11.8 ± 1.8	3.2 ± 3.1
Gomisin N	0.1	11.6 ± 1.3	18.4 ± 2.6★
	1.0	19.2 ± 1.9★	50.1 ± 3.2★★★,‡
	5.0	15.4 ± 1.2	15.5 ± 3.4
Wuweizisu C	0.1	11.0 ± 1.7	16.8 ± 1.7★
	1.0	31.0 ± 3.8★★	55.9 ± 1.9★★★,‡
	5.0	16.9 ± 2.4	21.2 ± 1.6★

†Cortical cultures were pretreated with the lignans for 1 hr before exposure to NMDA or KA. Cell viabilities of Ctrl and NMDA/KA-treated cells were representative as 100% and 0%, respectively. ODs of Ctrl, NMDA, and KA were 1.20 ± 0.10, 0.85 ± 0.08, and 0.77 ± 0.05, respectively. The values shown are the mean ± SEM of three experiments (five or six cultures per experiment). Asterisks indicate significance vs. excitotoxin-intoxicated cells.

[a]NMDA/KA-injured cells differ significantly from the control at $P < .001$.

★$P < .05$.

★★$P < .01$,

★★★$P < .001$.

‡$P < .01$ vs NMDA + each lignan (ANOVA and Tukey's test).

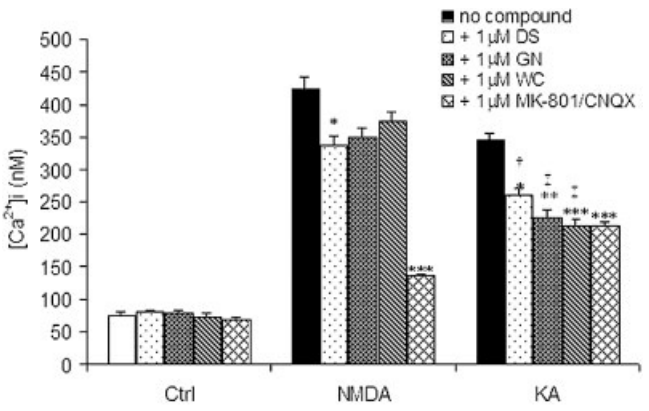


Fig. 5. Effects of deoxyschisandrin, gomisin N, and wuweizisu C on intracellular [Ca^{2+}] in NMDA- and KA-injured rat cortical cells. The values shown are the mean ± SEM of three experiments (five or six cultures per experiment). ★$P < .05$, ★★$P < .01$, ★★★$P < .001$ vs. excitotoxin-intoxicated cells; †$P < .05$, ‡$P < .001$ vs. NMDA + each lignan (ANOVA and Tukey's test).

glutathione defense system were not statistically significant.

We further determined that the level of cellular peroxide in glutamate-injured cortical cells was indeed reduced by treatment with wuweizisu C, gomisin N, and deoxyschisandrin (Table IV). At a concentration of 1 μM, wuweizisu C, gomisin N, and deoxyschisandrin significantly reduced the content of cellular peroxide generated in glutamate-injured cortical cells.

TABLE III. Effect of Deoxyschisandrin, Gomisin N and Wuweizisu C on Nitric Oxide and Cellular Peroxide Levels in Cortical Cell Cultures Injured by Glutamate†

	Nitrite (nM)	Cellular peroxide (arbitrary units)
Control	252.2 ± 13.1	95.5 ± 13.0
Glutamate-injured	1,104.2 ± 21.4	403.3 ± 10.4
Glutamate + deoxyschisandrin	724.3 ± 3.1★★	252.2 ± 20.7★
Glutamate + gomisin N	571.7 ± 3.3★★	228.8 ± 11.1★★
Glutamate + wuweizisu C	516.8 ± 2.9★★	192.5 ± 9.0★★

†Cortical cell cultures were pretreated with lignans (1 μM) 1 hr before exposure to 50 μM glutamate and then maintained for 24 hr in the presence of lignans (*treatment throughout*). The values shown are the mean ± SEM of three experiments (five or six cultures per experiment). Glutamate-injured cells differ significantly from the control of $P < .001$. Asterisks indicate significance *vs.* glutamate-intoxicated cells (ANOVA and Tukey's test).

★$P < .01$.

★★$P < .001$.

TABLE IV. Effect of Deoxyschisandrin, Gomisin N and Wuweizisu C on Glutathione Peroxidase Activity and the Content of Glutathione in Glutamate-Injured Cortical Cell Cultures†

	GSH-px (μmol NADPH consumed/min/mg protein)	GSH (nmol/mg protein)
Control	18.7 ± 1.7	6.9 ± 1.6
Glutamate-injured	9.2 ± 1.9★	2.6 ± 0.4
Glutamate + deoxyschisandrin	15.3 ± 1.7	4.7 ± 0.6
Glutamate + gomisin N	14.6 ± 2.4	4.8 ± 0.8
Glutamate + wuweizisu C	13.9 ± 2.3	4.4 ± 0.5

Cortical cell cultures were pretreated with lignans (1 μM) 1 hr before exposure to 50 μM glutamate and then maintained for 24 hr in the presence of lignans (*treatment throughout*). The values shown are the mean ± SEM of three experiments (four cultures per experiment).

★Glutamate-injured cells differ significantly from the control at $P < .01$ (ANOVA and Tukey's test).

DISCUSSION

This study was performed in an attempt to demonstrate pharmacological activity of active compounds isolated from natural products, especially neuroprotectivity, and to elucidate the mechanism(s) responsible. Even though natural products have been used for the treatment of several neurological disorders, such as stroke and ischemia, only limited scientific evidence on their traditional use and mechanism of action has been reported.

Although the five lignans (deoxyschisandrin, gomisin A, gomisin N, schisandrin, and wuweizisu C) isolated from *S. chinensis* had a similar structure with a dibenzocyclooctadiene skeleton, our present work demonstrates that only wuweizisu C, gomisin N, and deoxyschisandrin significantly attenuated glutamate-induced neurotoxicity in the treatment throughout paradigm at concentrations ranging from 100 nM to 5 μM (Fig. 3). We previously reported that well-known positive controls that inhibit glutamate-induced neurotoxicity, e.g., MK-801 (non-

competitive NMDA antagonist; 84% of neurons survived) and CNQX (non-NMDA antagonist; 62% of neurons survived), showed effective neuroprotective activity in our culture system at a concentration of 10 μM (Kim et al., 2002). The neuroprotective activity of wuweizisu C, gomisin N, and deoxyschisandrin at 5 μM was equipotent with that of 10 μM CNQX.

As regards structure–activity relationships, we postulate that the absence of a hydroxyl group at C-6 plays an important role in conveying neuroprotective activity, insofar as the introduction of a hydroxyl group at C-6 appears to reduce neuroprotective activity. It is known that, the more lipophilic a compound, the better it can penetrate cell membranes (Dearden, 1985). Deoxyschisandrin, gomisin N, and wuweizisu C, which have no hydroxyl group at C-6 in the dibenzocyclooctadiene structure, are more lipophilic than gomisin A and schisandrin, which possess a hydroxyl group at the same position.

Wuweizisu C, gomisin N, and deoxyschisandrin, which showed neuroprotection against glutamate-induced toxicity, have the same dibenzocyclooctadiene skeleton and differ only in the number of methylenedioxy groups (Fig. 1). Ip et al. (1997) reported that the methylenedioxy groups of these lignans play a crucial role in their antihepatotoxic activity against CCl_4. A similar pattern was observed in the present work. Treatment with wuweizisu C, which has two methylenedioxy groups, was more effective than treatment with gomisin N, which has only one methylenedioxy group. Deoxyschisandrin, which has no methylenedioxy group, was less effective than either wuweizisu C or gomisin N. These findings indicate that the presence of either a methylenedioxy group or a hydroxyl group on the dibenzocyclooctadiene skeleton is an important structural determinant for neuroprotective activity.

Although these lignans are different only in the number of methylenedioxy groups in their structures, they showed somewhat different protective effects in glutamate-injured cortical neurons. Pretreatment of deoxyschisandrin, which has no methylenedioxy groups, significantly attenuated glutamate-induced neurotoxicity more than posttreatment with the lignan. By contrast, posttreatment with wuweizisu C and gomisin N that have the methylenedioxy groups was more effective than pretreatment with these lignans. Interestingly, wuweizisu C could protect glutamate-damaged cortical neurons when administered 3 hr after glutamate exposure (Fig. 4). From these results, we suggest that wuweizisu C, gomisin N, and deoxyschisandrin have somewhat different neuroprotective modalities in our in vitro culture system, according to the number of methylenedioxy groups in their structure.

We could postulate that these three lignans might not directly act on NMDA receptors, because these lignans did not show significant neuroprotectivity against NMDA-induced toxicity. This suggestion was supported by separate results showing that deoxyschisandrin, gomisin N, and wuweizisu C had more potent neuroprotective activity in KA-induced neurotoxicity (Table II) and inhibited Ca^{2+} influx in KA-injured rat cortical cells (Fig. 5).

The inflow of calcium induced by excess glutamate is involved in the activation of phospholipases, with the release of membrane-derived molecules such as free arachidonic acid, eicosanoids, and platelet-activating factor (PAF; Coyle and Puttfarcken, 1993). These lipid messengers can modulate the activities of ion channels, receptors, and enzymes in the early neuronal responses to stimulation and may be converted into mediators of injury participating in glutamate-induced toxicity (Clark et al., 1992; Bazan et al., 2002). Lignans from *S. chinensis* were reported to be PAF antagonists (Jung et al., 1997; Lee et al., 1999), which could be expected to reduce Ca^{2+} influx in the CNS (Maclennan et al., 1996). Thus, PAF inhibitors such as ginkgolides A, B, and C can protect neurons against glutamate-induced neurotoxicity (Krieglstein et al., 1995). Among the three lignans, deoxyschisandrin showed the most potent PAF antagonism in the previous study (Jung et al., 1997). That is, these lignans' inhibition on Ca^{2+} influx evoked by KA in our culture system was not perfectly correlated with the PAF antagonism of *Schisanda* lignans. Therefore, we suggested that the effect of wuweizisu C and gomisin N on the increment of $[Ca^{2+}]_i$ induced by KA might primarily account for their neuroprotective activity against KA-induced neurotoxicity. However, we cannot fully exclude the possibility that these lignans protected cortical neurons via their PAF antagonism.

In spite of the failure to inhibit the increment of $[Ca^{2+}]_i$ induced by NMDA by treatment with all three lignans, wuweizisu C, gomisin N, and deoxyschisandrin significantly reduced NO production in our system (Table III). Furthermore, wuweizisu C, gomisin N, and deoxyschisandrin significantly reduced the content of cellular peroxide generated in glutamate-injured cortical cells at 1 μM (Table III). Wuweizisu C and gomisin N were previously reported to have direct free radical-scavenging activity (Li et al., 1990; Xue et al., 1992). The scavenging effects of wuweizisu C were stronger that those of gomisin N because of the numbers of methylenedioxy groups. In that the methyoxyl group repels electrons, but the methylenedioxy groups attracts electrons, there may be electron withdrawal from the phenyl rings and an area of high electron density on the methylenedioxy group, which may facilitate radical attack (Li et al., 1990).

In our cortical culture system, wuweizisu C, gomisin N, and deoxyschisandrin also attenuated the decrease in GSH-px activity and GSH content in cultures insulted with glutamate (Table IV). Although their effects on the glutathione defense system were not statistically significant, it is still possible that the enhancing effect of these three lignans on the antioxidative defense system also contributes to their neuroprotectivity against glutamate-induced neurotoxicity. We propose that wuweizisu C, gomisin N, and deoxyschisandrin reduced the formation of ROS in cells by enhancing the activity of the antioxidative defense system and free radical-scavenging activity;

this eventually prevented the formation of the peroxynitrite radical, a product of the reaction of NO and O_2^-.

Natural products and/or their synthetically developed active components have been used widely in medicine. *Gingko biloba* extract (Egb 761) is one of the best known natural products with effects on the CNS. Recent evidence indicates that Egb 761 protects against neuronal degeneration caused by oxidative stress (Oyama et al., 1993; Ni et al., 1996). At present, the cellular and molecular mechanisms that underlie the action of wuweizisu C, gomisin N, and deoxyschisandrin are not fully understood. However, our data clearly demonstrated the decrease in KA-induced Ca^{2+} entry, and the enhancement of the glutathione defense system by these three lignans might explain the neuroprotective effects observed in the present in vitro study. In conclusion, natural products that attenuate the influx of Ca^{2+} and further improve the antioxidative defense system might offer a useful therapeutic choice in the treatment of neurodegenerative disorders caused by oxidative stress.

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