Neuroprotective Effects of 2,5-Diaryl-3,4-dimethyltetrahydrofuran Neolignans

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We previously reported the neurotrophic effects of talaumidin (1) from *Aristolochia arcuata* MASTERS. In the present study, we compared the neurotrophic and neuroprotective effects of six other 2,5-diaryl-3,4-dimethyltetrahydrofuran neolignans isolated from the same plant, veraguensin (2), galgravin (3), aristolignin (4), nectandrin A (5), isonectandrin B (6), and nectandrin B (7), with compound 1 in primary cultured rat neurons. Compounds 3—7 promoted neuronal survival and neurite outgrowth, among which compounds 6 and 7 showed neurotrophic activity comparable with that of 1. Furthermore, compounds 1—7 protected hippocampal neurons against amyloid β peptide ($A\beta_{25—35}$)-induced cytotoxicity, while compounds 1 and 4—7 protected against neuronal death from 1-methyl-4-phenylpyridinium ion (MPP^+)-induced toxicity in cultured rat hippocampal neurons.

Key words 2,5-diaryl-3,4-dimethyltetrahydrofuran; neuroprotection; neurotrophic effect; primary cultured neuron; *Aristolochia arcuata*

Neuronal death contributes greatly to the brain deficits in neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and some ischemia-induced dementia.[1] Ideal treatment and prevention for these diseases would interrupt the degenerative procedure specific to each disease, such as the trial of a β-secretase inhibitor to reduce the production of toxic amyloid β peptides that play key roles in Alzheimer's disease.[2] However, there is still a long way to go to clarify their deleterious mechanisms, which may be initiated by toxic insults,[3] viral infection,[4] imbalance of nutrients,[5] genetic defaults,[6] natural aging [7] or complex interactions among them. Therefore offering protective agents to rescue damaged neurons from death is regarded as a practicable strategy for the control of neurodegenerative diseases.[8] Several compounds acting with different protective mechanisms are currently in development and a few are in clinical trials, including antioxidants,[9] vitamins,[10,11] endogenous neurotrophins,[12] and neurotrophin functional mimetics.[13] Our ongoing project to search for small-molecular functional mimetics of neurotrophins from natural products resulted in the discovery of a promising 2,5-diaryl-3,4-dimethyltetrahydrofuran neolignan, talaumidin (**1**), which was isolated from *Aristolochia arcuata* MASTERS and was confirmed to promote neurite outgrowth and neuronal survival in cultured rat cortical neurons.[14] Our continued work led to the isolation of six previously known neolignans (**2**—**7**) (Fig. 1) from a methanol extract of the same plant. In this paper, we report the neurotrophic effects of these 2,5-diaryl-3,4-dimethyltetrahydrofuran neolignans (**2**—**7**) for comparison with **1**, the neuroprotective effects of neolignans (**1**—**7**) against $A\beta_{25—35}$-induced cell death in primary cultured rat hippocampal neurons,[15] as well as against cell death induced by 1-methyl-4-phenylpyridinium ion (MPP^+), a neurotoxin used in the cellular model for Parkinson's disease.[16]

MATERIALS AND METHODS

Materials Compound **1**[17] and its analogues, veraguensin (**2**),[18] galgravin (**3**),[19] aristolignin (**4**),[19] nectandrin A (**5**),[20] isonectandrin B (**6**),[20] and nectandrin B (**7**),[20] were isolated from a methanol extract of the root of *A. arcuata* as previously described.[14] Their structures were elucidated by extensive analyses of IR, MS, ^{1}H-, and ^{13}C-NMR spectra and identified by spectral data and specific rotations in the literature.[17—20] Cell culture medium and supplements were from Gibco BRL Co. Ltd. (NY, U.S.A.). Cell culture plates were purchased from Iwaki (Chiba, Japan). Human recombinant basic fibroblast growth factor (bFGF) was from Upstate Biotechnology, Inc. (NY, U.S.A.); thiazolyl blue tetrazolium bromide (MTT) was from Sigma (MO, U.S.A.); Tau (V-20) anti-tau antibody was from Santa Cruz Biotechnology, Inc.

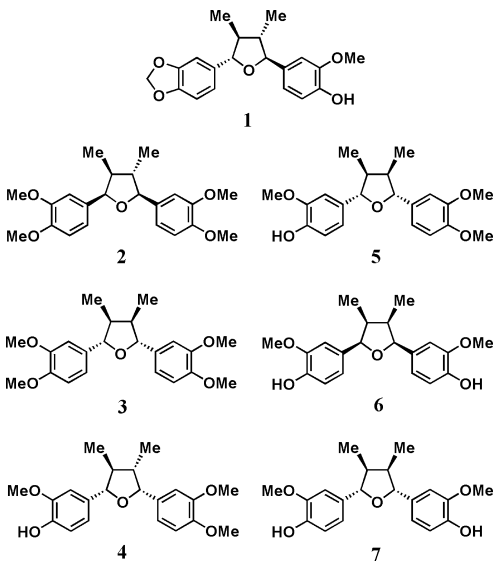

Fig. 1. Chemical Structures of Compounds **1**—**7**

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(CA, U.S.A.); second antibody SimpleStain PO (rabbit anti-mouse IgG) and SimpleStain DAB solution were from Nichirei Corporation (Tokyo, Japan). Stock solution of amyloid β protein (human, A$\beta_{25—35}$, from Peptide Institute Inc., Osaka, Japan) was prepared with double-distilled water to 200 μM and aggregated at 37 °C for 48 h. Stock solution of MPP^+ (Sigma) was prepared with Dulbecco's phosphate-buffered saline (PBS) to a concentration of 20 mM.

Cell Culture Primary cortical and hippocampal neuronal cultures were derived from embryonic day 18 Sprague-Dawley rat pups (Japan SLC, Inc.) as previously described.[14] Briefly, cerebral neocortices and hippocampi were removed, taking care to avoid the olfactory bulbs. The tissue was dissected, minced with forceps, and then completely dissociated into a single-cell suspension using trypsin digestion. Isolated neurons were diluted to the desired concentration, seeded on poly-L-lysine (Sigma)-coated culture plates in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin 50 IU/ml and streptomycin 50 μg/ml. The cells were maintained at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 until used.

Neurite Outgrowth Assay Freshly isolated cortical neurons were cultured in 24-well plates at a density of 5000/cm^2 for 24 h; then the culture medium was changed from DMEM/10% FBS to Neurobasal/2% B27 supplement with or without test compounds. After further 72-h culture, the neurons were fixed with 4% paraformaldehyde in Dulbecco's PBS for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 20 min after endogenous peroxidase activity was blocked by freshly prepared 0.3% H_2O_2 for 20 min. Neurons were then incubated in primary antibody anti-tau Tau (v-20) (1 : 500) overnight at 4 °C, followed by incubation with peroxidase-conjugated second antibody SimpleStain PO (1 : 2) for 1 h. Peroxidase was developed with 200 μl substrate of SimpleStain DAB. Neurons were imaged and analyzed for the length of the primary (longest) neurite with a software LuminaVision 1.0/MacScope 2.6 system (Mitani Corp., Fukui, Japan).

Neuronal Viability Measurement Neuronal viability was determined in the MTT reduction assay as previously described.[21] Briefly, neurons were cultured in 96-well plates. MTT (0.5 mg/ml in PBS) was added to the culture medium (MTT : medium = 1 : 10); after 3-h incubation, the medium was pipetted off, and 50% ethanol/50% dimethyl sulfoxide was used to dissolve the MTT formazan. Then absorbance was measured at 560 nm (reference 650 nm) and translated into neuronal viability.

Statistical Analysis Data were analyzed with software Origin 7.0 (OriginLab Corp., MA, U.S.A.). All data are presented as mean±S.E. The significance of differences among groups was determined using one-way ANOVA followed by group mean comparison with the Bonferroni *post hoc* test or Student's *t*-test.

RESULTS

Neurotrophic Effects in Cultured Cortical Neurons Together with **1**, six previously known 2,5-diaryl-3,4-dimethyltetrahydrofuran neolignans, **2**—**7** were isolated from *A. arcuata* MASTERS (Fig. 1).

Since we found that **1** has intriguing neurotrophic activities, such as neurite outgrowth promotion and neuronal survival promotion in cultured rat cortical neurons, we examined the neurotrophic effects of these tetrahydrofuran-type neolignans (**2**—**7**) in the same assays as used for the evaluation of **1**.[14] In the present study, compounds **3**—**7** showed protective effects against serum withdrawal-induced neuronal death in the dose range of 1—30 μM (Table 1).

As compared with **1** and the positive control bFGF 10 ng/ml, compounds **6** and **7** showed a similar degree of protective activity as **1** at 10 μM, while compounds **3**, **4**, and **5** showed less protective effects than **1** (Fig. 2). In the neurite outgrowth assay, compounds **5**, **6**, and **7** were similar in activity to **1**, but compounds **3** and **4** showed fewer effects than **1**, although compound **1** had significant effects in the dose range of 10—30 μM (Fig. 3). Thus compounds **6** and **7** showed as potent neurotrophic activities as **1** not only in the model of serum withdrawal-induced cell death but also in the neurite outgrowth assay (Figs. 2, 3).

Neuroprotection against A$\beta_{25—35}$-Induced Neuronal Death in Hippocampal Neurons Since these tetrahydrofuran-type neolignans (**1**—**7**) showed promising neurotrophic effects in cultured rat cortical neurons, we further investigated their neuroprotective effects against A$\beta_{25—35}$ induced-neuronal death in primary cultured rat hippocampal neurons. In our preliminary experiment, A$\beta_{25—35}$ dose dependently induced cell death in cultured hippocampal neurons at concentrations higher than 5 μM (data not shown). The morphology of hippocampal neurons cultured for 72 h showed indications of good viability, including healthy soma and long neurites (Fig. 4A). Treatment of neurons with A$\beta_{25—35}$ led to dramatically detrimental morphology and caused cell death.

Table 1. Effect of Compounds **1**—**7** on the Cell Survival after Serum Deprivation in Cultured Rat Cortical Neurons

Compounds	Neuronal survival under various concentration				
	0 μM	1 μM	3 μM	10 μM	30 μM
Talaumidin (**1**)[14]	100.00±4.18	120.67±8.82	142.21±16.19*	194.12±17.53***	180.83±9.71**
Veraguensin (**2**)	100.00±2.25	110.99±3.19	116.38±4.91	112.13±4.89	120.39±5.63
Galgravin (**3**)	100.00±2.25	117.20±4.63	137.41±6.18*	139.18±6.42**	121.45±3.89
Aristolignin (**4**)	100.00±5.13	104.19±3.18	116.53±4.82*	144.23±4.75***	164.56±5.68***
Nectandrin A (**5**)	100.00±5.13	106.44±1.78	110.36±2.61	151.16±4.66***	166.22±6.46***
Isonectandrin B (**6**)	100.00±7.54	121.78±5.90	133.52±6.47*	194.32±4.93***	230.49±9.92***
Nectandrin B (**7**)	100.00±4.78	133.33±4.86	153.60±6.99***	204.17±12.52***	264.20±8.54***

Rat cortical neurons were first cultured in DMEM/10% FBS at a density of 2.0×10^5/cm^2 for the first 24 h, and then culture medium was changed to serum-free DMEM containing 1% N2 supplement. After a further 48 h, neuronal viability was determined in the MTT reduction assay. Data are expressed as mean±S.E. (% of control, $n=6$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control.

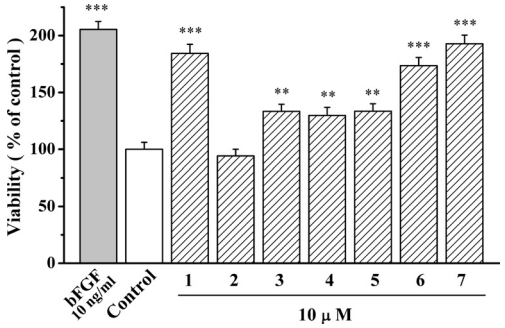

Fig. 2. Comparison of the Effects of Compounds **1**—**7** on Cell Survival after Serum Deprivation in Primary Cultured Rat Cortical Neurons

The method is described in the notes to Table 1. bFGF 10 ng/ml was used as a positive control. Data are expressed as mean±S.E. (n=6). ** p<0.01; *** p<0.001 *vs.* control.

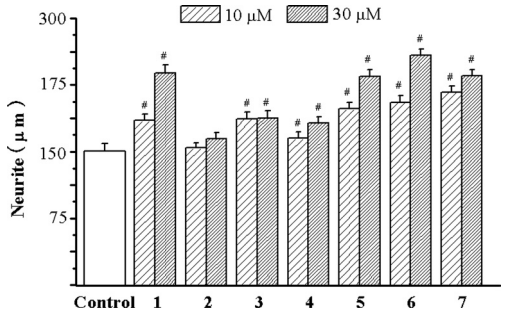

Fig. 3. Comparison of the Effects of Compounds **1**—**7** on Neurite Outgrowth in Primary Cultured Rat Cortical Neurons

The method is described in Materials and Methods. Data are expressed as mean±S.E. (n=80). # p<0.001 *vs.* control.

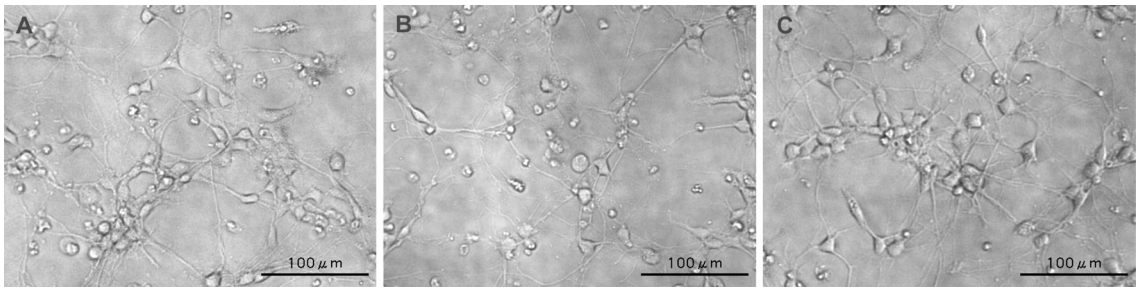

Fig. 4. Effects of A$\beta_{25—35}$ and **1** on Morphologic Integrity of Cultured Hippocampal Neurons

Rat hippocampal neurons were cultured in DMEM/10%FBS for 24 h, and then medium was changed to serum-free DMEM containing 1% N2 supplement. At the same time, A$\beta_{25—35}$ was added in the presence or absence of test samples. After 48-h treatment, neurons were imaged under a reverse microscope equipped with a CCD camera. A, control neurons; B, neurons treated with A$\beta_{25—35}$ 10 μM; C, neurons treated with A$\beta_{25—35}$ 10 μM and compound **1** 10 μM.

After A$\beta_{25—35}$ exposure for 48 h, many dead neurons appeared in each field when observed under a microscope; neuritic processes became shortened, damaged, or disappeared (Fig. 4B).

When the hippocampal neurons were exposed to 10 μM A$\beta_{25—35}$ in the presence of compound **1** 10 or 30 μM, the neurons maintained healthy integrity (Fig. 4C). The percentage of surviving neurons after 48-h exposure to **1** and **2**—**7** under the various conditions was estimated using MTT reduction assay (Fig. 5). Neurons treated only with **1** dose dependently increased neuronal viability (Fig. 5A), whereas 10 μM A$\beta_{25—35}$ caused a 35% decrease in neuronal viability compared with the control. The presence of **1** dose dependently protected against neuronal death from A$\beta_{25—35}$-induced toxicity (Fig. 5A). In comparison of **1** with **2**—**7**, compounds **2**—**7** showed good protection against A$\beta_{25—35}$-induced neuronal death in that order, with minimum effects of compound **2** to maximum effects of compound **7** (Fig. 5B).

Neuroprotection against MPP$^+$-Induced Neuronal Death in Hippocampal Neurons The protective effects of **1** and its analogues **2**—**7** were further examined against MPP$^+$-induced toxicity in cultured rat hippocampal neurons. MPP$^+$ decreased neuronal viability in the cultured hippocampal neurons at concentrations of 50—200 μM (Fig. 6A). When MPP$^+$ 100 μM was used to induce neuronal death, **1** enhanced the neuronal viability dose dependently at 1—30 μM (Fig. 6A), and compounds **4**—**7** also showed various protective effects at 10 μM (Fig. 6B).

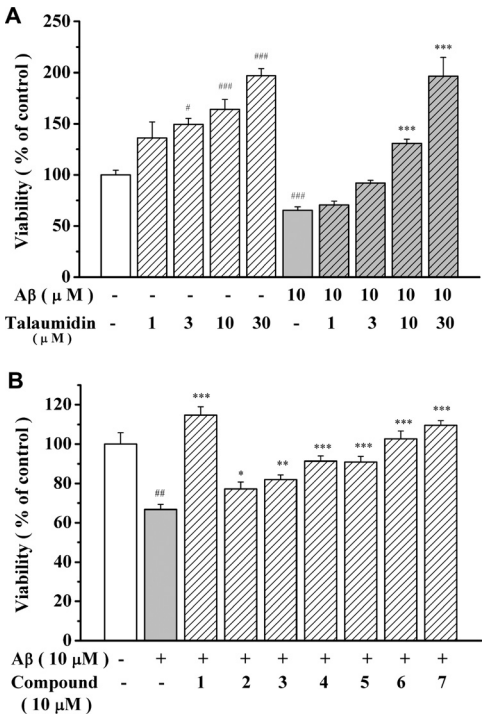

Fig. 5. Protective Effects of Compounds **1**—**7** on Neuronal Survival after A$\beta_{25—35}$ Treatment in Hippocampal Neurons

Neurons were cultured and treated as described in the legend to Fig. 4. After 48-h treatment, neuronal viability was determined in the MTT reduction assay. Data are expressed as the mean±S.E. (n=6). # p<0.05; ## p<0.01; ### p<0.001 compared with control; * p<0.05; ** p<0.01; *** p<0.001 compared with A$\beta_{25—35}$ 10 μM.

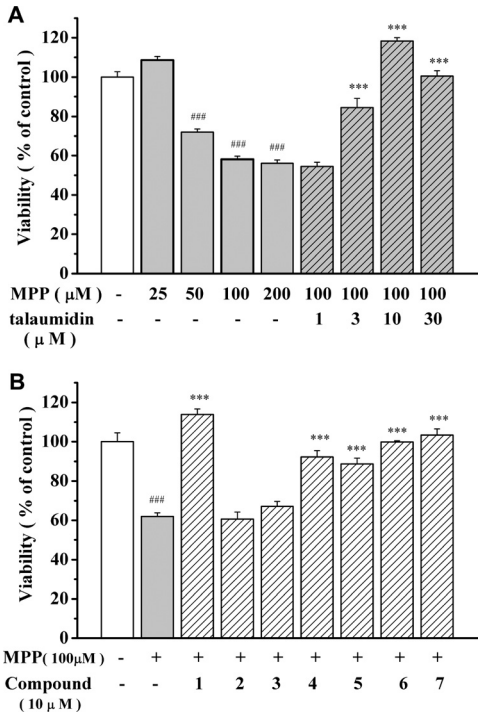

Fig. 6. Effects of Compounds **1**—**7** on Neuronal Survival in Cultured Hippocampal Neurons after MPP^+ Injury

Neurons were cultured in DMEM/10% FBS for 24 h, and then medium was changed to serum-free DMEM containing 1% N2 supplement. At the same time, MPP^+ was added in the presence or absence of test samples. After 48-h treatment, neuronal viability was determined in the MTT reduction assay. Data are expressed as the mean±S.E. (n=6). ###p<0.001 compared with control; ***p<0.001 compared with MPP^+ 100 μM.

DISCUSSION

In this study, we evaluated the neurotrophic and neuroprotective effects of six 2,5-diaryl-3,4-dimethyltetrahydrofuran neolignans (**2**—**7**) isolated from *A. arcuata* in comparison with those of **1**. Although this type of neolignan is widely distributed in plants, there are few reports on their biological activities, which include trypanocidal activity,[22] anti-platelet-activating factor activity,[23] and neuroprotective activity of galbelgin against glutamate-induced toxicity in cultured cortical neurons.[24] The intriguing neurotrophic activity of **1**[14] prompted us to compare **1** with its analogues **2**—**7** from *A. arcuata*. Our results indicated that except for compound **2**, the other compounds showed different levels of neurotrophic effects in the model of serum withdrawal-induced neuronal death (Table 1, Fig. 2) and neurite outgrowth assay (Fig. 3). According to the present results, the *para*-substituted phenolic hydroxyl groups on two benzene rings are most likely to be essential for the neurotrophic activity of these neolignans, but the number of hydroxyl groups is not a crucial factor, because **1** with one hydroxyl group exhibited the most potent neurotrophic activity among the tested neolignans. Compound **1** has two benzene rings substituted in the *trans* configuration on a 1,2-dimethyltetrahydrofuran ring, whereas the other compounds **2**—**7** have a *cis* relationship for two benzene rings. The above preliminary struture–activity analysis assume that the configuration of two benzene substituents on a tetrahydrofuran ring would make an important contribution to the enhancement of activity. However, the role of 1,2-dimethyl groups in a tetrahydrofuran remains ambiguous at present.

Next, we examined the neuroprotective effects of **1**—**7** using two *in vitro* models of neurodegenerative diseases. Compounds **1**—**7** showed protection against A$\beta_{25—35}$-induced hippocampal neuronal death. As shown in Fig. 5A, **1** has protective effects against cell death, which is presumably caused by complete medium exchange-induced cellular stress. In the presence of 30 μM of **1**, the neuronal viability in the A$\beta_{25—35}$-treated groups is comparable to that in A$\beta_{25—35}$-untreated groups (196.6±18.3% *vs.* 197.0±6.7%), indicating that it can override the damage caused by A$\beta_{25—35}$ insult. MPP^+, a mitochondria complex I inhibitor inducing Parkinson syndromes *in vivo*, is known to induce apoptosis in several neuronal cell types, such as cerebellar granule cells,[25] PC12 cells,[26] and SH-SY5Y cells,[27] and thus can be used to make an *in vitro* model of Parkinson's disease. Our results demonstrated that MPP^+ also shows toxic effects in the culture of rat hippocampal neurons at the dose range of 25—200 μM (Fig. 5A), and its toxicity was overcome by compounds **1** and **4**—**7** (Fig. 5B), assuming that hippocampal neurons could serve as a cellular model for MPP^+-induced toxicity closely related to Parkinson's disease, although MPP^+ typically injuries dopaminergic neurons.[28] Thus the protective effects of **1** and its analogues **4**—**7** on the MPP^+-induced toxicity of hippocampal neurons would allow exploring the possibility that 2,5-diaryl-3,4-dimethyltetrahydrofuran neolignans could be developed as neuroprotective agents.

In the present study, we found that **1** and its analogues **5**—**7** have neuroprotective effects in *in vitro* models of Alzheimer's disease and Parkinson's disease. 2,5-Diaryl-3,4-dimethyltetrahydrofurans can be regarded as potential prototypes to find new biological properties associated with this particular skeleton and develop new drugs for the treatment of neurodegenerative diseases. However, we do not have sufficient data to figure out the detailed structure–activity relationship and neuroprotective mechanism of **1**. Our study is now directed toward stereocontrolled syntheses of **1** and its possible stereoisomers.

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