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Dammarane Derivatives Protect Cultured Rat Cortical Cells from Glutamate-induced Neurotoxicity

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Abstract

We previously reported that ginsenosides Rb_1 and Rg_3, dammarane glycosides, of *Panax ginseng* C. A. Meyer (Araliaceae), significantly attenuated glutamate-induced neurotoxicity in primary cultures of rat cortical cells. To seek more potent neuroprotective compounds, we attempted to modify the chemical structure of dammarane glycosides and obtained six derivatives, MA-11, PT-11, PT-111, POA-101, POA-111 and N-001. The neuroprotective activity of these dammarane derivatives were evaluated employing primary cultures of rat corticoid cells.

The glutamate-induced neuronal cell damage was significantly reduced by a pre-treatment with protopanaxadiol, MA-11 or PT-11 at concentrations ranging from 100 nM to 10 μM. Both MA-11 and PT-11, preserved the levels of catalase and inhibited decreases in glutathione reductase in glutamate-injured cells. Furthermore, the dammarane derivatives reduced the content of intracellular peroxide in glutamate-intoxicated cells. Finally, they inhibited the formation of malondialdehyde, a compound produced during lipid peroxidation, in glutamate-insulted cells.

These results show that the dammarane derivatives, MA-11 and PT-11, exert significant neuroprotective effects on cultured cortical cells by a mechanism seemingly distinct from that afforded by ginsenosides Rb_1 and Rg_3. As such, the dammarane derivatives may be efficacious in protecting neurons from oxidative damage caused by exposure to excess glutamate.

Glutamate plays a dominant role in central excitatory neurotransmission and participates in neuronal cell loss in the CNS (Choi 1988; Choi et al 1989). It has been reported that excitatory amino acids, including glutamate, are involved in neuronal survival, synaptogenesis, neuronal plasticity and the learning and memory processes (Monaghan et al 1989; Kruk & Pycock 1991). Abnormalities in excitatory amino-acid neurotransmitter systems may be involved in neurological disorders such as seizures (Zaczek & Coyle 1982), Alzheimer's disease (Greenamyre & Young 1989), ischaemia and spinal cord trauma (Kariman 1985; Alber et al 1989).

The root of *Panax ginseng* C. A. Meyer (Araliaceae) has been used as a tonic for several thousand years in China, Korea and Japan (Cho et al 1995). Ginseng is known to increase arousal, stamina and resistance to stress (Takagi et al 1972a; Saito et al 1974; Cho et al 1995). Its stimulant effects on the CNS, action against fatigue and enhancement of non-specific resistance have been attributed to the saponin constituents (Takagi et al 1972b; Nabata et al 1973). Thus, we initially evaluated the neuroprotective activity of the saponin constituents using glutamate-insulted primary cultured rat cortical cells as an in-vitro system, and previously reported that ginsenosides Rb_1 and Rg_3, protopanaxadiol components of *P. ginseng*, significantly attenuated glutamate-induced neurotoxicity in primary cultured corticoid cells (Kim et al 1998). However, neuroprotective effects of ginsenosides Rb_1 and Rg_3 were reduced at concentrations above 10·0 μM.

In this study, we modified the chemical structure of protopanaxadiol (PPD) and protopanaxatriol (PPT) to improve the neuroprotective activity or reduce the cytotoxic effects of authentic ginseno-

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sides. Primary cultures of rat cortical cells were used to evaluate the neuroprotective effects of these compounds. Among the 6 derivatives tested (MA-11, PT-11, PT-111, POA-101, POA-111 and N-001), only MA-11 and PT-11 showed neuroprotective activity equivalent to that of ginsenosides Rb_1 and Rg_3 against neuronal damage induced by excess glutamate.

Materials and Methods

Preparation of dammarane derivatives

20-(*S*)-protopanaxadiol (20-(*S*)-PPD) and 20-(*S*)-protopanaxatriol (20-(*S*)-PPT), aglycones of dammarane glycosides, were purified from *P. ginseng*, which was purchased from Kyongdong herbal market. Other chemicals were purchased from Aldrich Co. The dammarane derivatives were synthesized by chemically modifying PPD or PPT. 20-(*S*)-PPD (for MA-11 and PT-11) or 20-(S)-PPT (for PT-111, POA-101, POA-111 and N-001) was dissolved in 2 mL anhydrous CH_2Cl_2, anhydrous pyridine, and either *m*-anisoyl chloride (for MA-11), *p*-toluoyl chloride (for PT-11 and PT-111), phenoxyacetyl chloride (for POA-101 and POA-111) or nicotinoyl chloride·hydrochloride (for N-001), and stirred magnetically at room temperature for 30 min. After the reactions, the solutions were quenched by stirring with H_2O. The solutions were diluted in 60 mL CH_2Cl_2 and partitioned twice with the same volume of 0·01 M NaOH and 0·01 M HCl, respectively. The resultant CH_2Cl_2 fractions were evaporated in-vacuo and chromatographed on a silica-gel column eluted with step-gradients from $CHCl_3$–CH_3OH (100 : 1→30 : 1) to give 6 derivatives, MA-11, PT-11, PT-111, POA-101, POA-111 and N-001.

Cell culture

Primary cultures of mixed cortical cells containing both neuronal and glial cells were prepared from 17–19-day-old foetal Sprague-Dawley rats as described previously (Kim et al 1998). The trypsin-dissociated cortical cells were plated onto 15-mm dishes (Falcon) coated with poly-L-lysine at a density of 1×10^5 cells/dish (for the estimation of cellular oxidation and cell viability using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) and released lactate dehydrogenase, LDH) or 60-mm dishes coated with collagen (Falcon) at a density of 3×10^6 cells/dish (for the estimation of nitric oxide, NO content, antioxidative enzymes activity and lipid peroxidation). Cultures were allowed to mature for 2 weeks.

Neurotoxicity

All dammarane derivatives were dissolved in dimethylsulphoxide (DMSO; final culture concentration 0·1%; Junsei Chemical Co). Preliminary studies indicated that the solvent had no effect on cell viability at the concentration used. Cortical cell cultures were washed with DMEM (Dubecco's Modified Eagles Medium) and pre-incubated with dammarane derivatives for 1 h. The cultures were then exposed to 50 μM glutamate for 24 h. After exposure to glutamate, neuronal viability was quantified by measuring dehydrogenase activity retained in living cells using the MTT assay (Mosmann 1993; Kim et al 1998). Neuronal integrity was assessed by spectrophotometric measurement of the efflux of lactate dehydrogenase (LDH) into the culture medium (Kim et al 1998). To assess neuronal integrity, 30 μL of the medium was collected and assayed for LDH release by the modified method of Choi & Koh (1987). Data were expressed as the percentage of viable cells relative to the vehicle-treated control cultures.

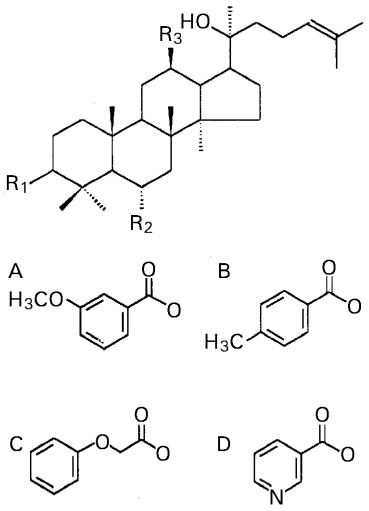
Intracellular calcium $[Ca^{2+}]_i$

Intracellular free calcium, $[Ca^{2+}]_i$, was determined using the calcium-sensitive compound Fura-2 AM (Molecular Probes, OR; Sigma) by ratio fluorometry as described previously (Kim et al 1998). Cells grown on glass cover slides were loaded with 5 μM Fura-2 AM for 1 h in phosphate-buffered saline (PBS, pH 7·2) at 37°C in a humidified atmosphere of 95% air–5% CO_2, and then washed with PBS. Cell-culture slides were cut and mounted into spectrophotometer cuvettes containing 2·5 mL PBS (without bicarbonate). Fluorescence was measured with a spectrophotofluorometer by exciting cells at 340 and 380 nm and measuring light emission at 520 nm. One hour before exposure to 50 μM glutamate, cultures were treated with dammarane derivatives. The $[Ca^{2+}]_i$ was measured 30 min after exposure to glutamate. Calcium concentrations were calculated according to the method of Grynkiewicz et al (1985).

Nitrite content

The level of NO formed was determined by measuring the content of nitrite released into the culture medium by the method of Dawson et al (1994). The concentration was determined against a nitrite standard curve.

Table 1. Structures of dammarane derivatives.



Compound	R_1	R_2	R_3
PPD	OH	H	OH
PPT	OH	OH	OH
MA-11	A	H	A
PT-11	B	H	B
PT-111	B	B	B
POA-101	C	OH	C
POA-111	C	C	C
N-001	OH	OH	D

Antioxidative enzyme activity

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 3000 *g* at 4°C and the supernatant, consisting of the cytosolic and mitochondrial fractions, was used in enzyme assays. The activity of the antioxidative enzymes, superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-px) and glutathione reductase (GSSG-R), were assayed by our modified spectrophotometric methods (Kim et al 1998; Lee et al 2000).

Cellular oxidation

The relative levels of free radicals (i.e. peroxide) in cultured cells were measured using the oxidation-sensitive compound 2,7-dichlorofluorescin diacetate (DCF; Sigma) by the method of Goodman & Mattson (1994). Cells were loaded (50 μM, 50-min incubation), followed by 3 washes in HBSS (Hanks balanced salt solution) and then measured DCF fluorescence by exciting cells at 485 nm and measuring light emission at 530 nm.

Lipid peroxides

The content of malondialdehyde (MDA), a compound produced during lipid peroxidation, was determined using the thiobarbituric acid method (Yagi et al 1976). The concentration of malondialdehyde was determined against a 1,1,3,3-tetraethoxypropane standard curve.

Protein assay

The content of protein in cells was measured by the method of Lowry et al (1951).

Data analysis

Data were evaluated for statistical significance by an analysis of variance test using a computerized statistical package. The data were considered to be statistically significant if $P < 0.05$.

Table 2. The effect of dammarane derivatives on glutamate-induced neurotoxicity in primary cultures of rat cortical cells.

	Concn (μM)	Cell viability (%)	LDH released (mUnits mL^{-1})
Control		100·0 ± 4·2	105·9 ± 8·3
Glutamate[a,b]		0·0 ± 1·3	197·6 ± 10·6
PPD	0·10	43·3 ± 2·3**	147·3 ± 4·9**
	1·00	65·5 ± 1·9***	137·9 ± 1·5***
	10·0	37·4 ± 6·2*	157·8 ± 3·1*
MA-11	0·10	33·7 ± 1·5	165·0 ± 5·5
	1·00	65·6 ± 2·1***	145·1 ± 2·9***
	10·0	63·3 ± 3·2***	151·8 ± 2·2***
PT-11	0·10	38·7 ± 1·3	162·1 ± 8·8
	1·00	66·6 ± 1·5***	136·5 ± 2·1***
	10·0	57·0 ± 6·2**	145·3 ± 1·8**
PPT	0·10	5·3 ± 1·3	–
	1·00	7·7 ± 1·5	182·6 ± 2·9
	10·0	−48·3 ± 6·2	–
N-001	0·10	20·3 ± 4·5	173·8 ± 4·1
	1·00	10·0 ± 3·3	188·9 ± 5·9
	10·0	−25·3 ± 6·1	220·8 ± 5·1
PT-111	0·10	6·5 ± 2·0	191·6 ± 2·7
	1·00	26·4 ± 4·5	176·5 ± 4·8
	10·0	2·5 ± 6·2	193·1 ± 1·5
POA-101	0·10	28·0 ± 1·3	143·3 ± 5·5
	1·00	52·1 ± 1·5**	151·8 ± 2·9**
	10·0	41·4 ± 6·2*	146·7 ± 3·5*
POA-111	0·10	30·0 ± 2·2*	164·0 ± 8·5
	1·00	32·6 ± 1·8*	164·0 ± 2·7*
	10·0	29·7 ± 3·3*	166·2 ± 2·2*

[a]Cortical cell cultures were washed with DMEM and incubated with dammarane derivatives for one hour. The cultures were then exposed to 50 μM glutamate. After 24 h incubation in DMEM in the presence of dammarane derivatives, the cultures were assessed for the extent of neuronal damage. The values shown are the mean ± s.e.m. of three experiments. [b]Optical densities (OD) of untreated control and glutamate-treated cultures were 1·03 ± 0·08 and 0·58 ± 0·02, respectively. Cell viability was calculated as 100 × (OD of glutamate + dammarane-treated − OD of glutamate-treated)/(OD of control − OD of glutamate-treated). [c]Glutamate-treated value differs significantly from the untreated control ($P < 0.001$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with glutamate alone.

Results

We prepared six dammarane derivatives by chemically modifying PPD or PPT, MA-11 and PT-11 from PPD, PT-111, POA-111, POA-101 and N-001 from PPT (Table 1). We then evaluated the neuroprotective activity of these semi-synthetic compounds, as well as PPD and PPT as aglycones. All of the compounds were initially screened by assessing their ability to preserve the structural integrity and viability of cortical cells after treatment with glutamate.

PPD and the PPD derivatives, MA-11 and PT-11, significantly preserved cellular integrity as measured by a reduction in the release of LDH from glutamate-treated cells. The compounds also preserved the viability of glutamate-treated rat cortical cells as assessed by the MTT assay (Table 2). MA-11 and PT-11 protected neurons from glutamate-induced damage to a significant degree at concentrations ranging from 100 nM to 10·0 μM. Furthermore, PPD, MA-11 and PT-11 prolonged cell survival significantly up to 60 h at a concentration of 1 μM (Figure 1). However, the neuroprotective effect of these dammarane derivatives was not superior to that of the ginsenosides Rb_1 and Rg_3, as we previously reported (Kim et al 1998).

Of the four PPT derivatives, POA-101 and POA-111 significantly protected cultured rat cortical cells from neurotoxicity caused by excess glutamate at concentrations ranging from 100 nM to 10·0 μM (Table 2). The neuroprotective activity of POA-101 was significantly more potent than that of POA-111. The other PPT derivative, PT-111 did not show as great a neuroprotective effect as the two PPT derivatives mentioned above. However, this compound still mitigated the cytotoxic effects of glutamate. The heterocyclic derivative, N-001, did not significantly affect glutamate-induced neurotoxicity.

Changes mediated by Ca^{2+} influx and increased NO formation through glutamate receptor activation lead to neuronal degeneration via oxidative stress (Coyle & Puttfarcken 1993). As such, we examined the effects of MA-11 and PT-11 on Ca^{2+} influx and NO over-production induced by high concentrations of glutamate. Exposure to 50 μM glutamate caused a significant increase in intracellular Ca^{2+} and NO levels in cortical cell cultures (Table 3). MA-11 and PT-11, at a concentration of 1 μM, slightly reduced the calcium influx caused by glutamate. However, these PPD derivatives showed little effect on the inhibition of NO over-production by glutamate.

Since oxidative stress is well-known as a mechanism responsible for glutamate-induced neuronal degeneration (Coyle & Puttfarcken 1993), we determined the activity of the antioxidative enzymes, SOD, catalase, GSH-px and GSSG-R in glutamate-treated cells. Glutamate treatment markedly reduced the activity of SOD, catalase and GSH-px in cortical cultures (Table 4). Pre-treatment with MA-11 or PT-11, at a concentration of 1 μM, significantly attenuated the decrease in catalase activity in glutamate-treated cortical cultures (Table 4). Catalase activity in cultures treated with glutamate plus MA-11 or PT-11 fell slightly, while cultures treated with glutamate alone experienced a precipitous drop in catalase activity. Furthermore,

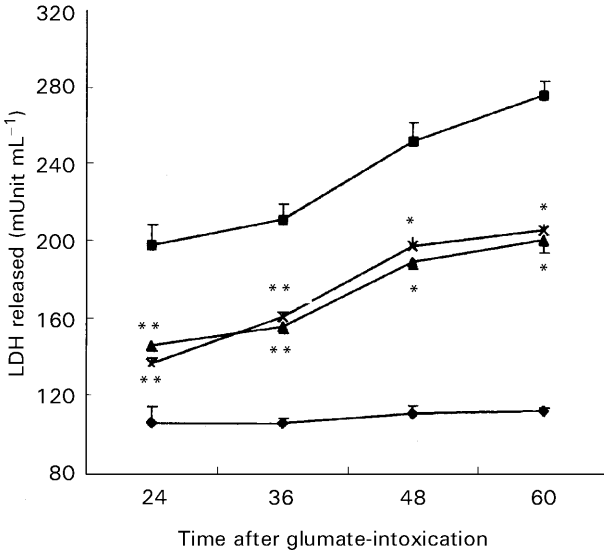


Figure 1. Prolonged neuroprotective effect of MA-11 or PT-11 on glutamate-induced neurotoxicity in primary cultures of rat cortical cells. Cortical cultures were treated with dammarane derivatives (1 μM) for one hour before glutamate-induced neurotoxicity. The values shown are the mean ± s.e.m. of three experiments. Control, ◆; glutamate-treated cells, ■; glutamate + MA-11, ▲; glutamate + PT-11, ×. Glutamate-treated values differ significantly from the untreated control ($P < 0.001$). *$P < 0.05$, **$P < 0.01$, compared with glutamate alone.

Table 3. Effect of MA-11 and PT-11 on intracellular Ca^{2+}, nitrite released and total glutathione in glutamate-treated cortical cell cultures[a].

	Intracellular Ca^{2+} (nM)	Nitrite (nM)	Total glutathione (μM)
Control	75·0 ± 15·0	453·5 ± 16·2	22·8 ± 2·7
Glutamate[b]	423·0 ± 20·0	1474·2 ± 31·2	6·3 ± 2·8
MA-11	311·6 ± 10·9*	1040·4 ± 18·6*	8·2 ± 1·3
PT-11	325·6 ± 9·6*	1167·5 ± 15·6	10·5 ± 2·7

[a]Cortical cultures were treated with dammarane derivatives (1 μM) for one hour before glutamate-induced neurotoxicity. The values shown are the mean ± s.e.m. of three experiments. [b]Glutamate-treated value differs significantly from the untreated control ($P < 0.001$). *$P < 0.05$, **$P < 0.01$, compared with glutamate alone.

MA-11 and PT-11 also significantly blocked the decrease in GSSG-R activity in glutamate-treated cultures (Table 4). However, these PPD derivatives had no significant effect on the activity of either SOD or GSH-px or on the content of total glutathione in glutamate-treated cultures (Tables 3 and 4).

Since oxidative stress causes lipid peroxidation (Coyle & Puttfarcken 1993), we further investigated the effects of MA-11 and PT-11 on the content of intracellular peroxide and cellular malondialdehyde, a compound produced by lipid peroxidation, in glutamate-treated cultures. Treatment with MA-11 and PT-11 reduced the intracellular peroxide in glutamate-injured cells (Table 5). Furthermore, these dammarane derivatives, at a concentration of 1 μM, reduced the content of cellular malondialdehyde significantly in glutamate-treated cultures (Table 5).

Table 5. Effect of MA-11 and PT-11 on intracellular peroxide and malondialdehyde in glutamate-treated cortical cell cultures[a].

	Intracellular peroxide (arbitrary units)	Malondialdehyde (pmol (mg protein^{-1}))
Control	84·6 ± 13·0	80·9 ± 5·2
Glutamate[b]	571·1 ± 10·4	216·3 ± 19·2
MA-11	353·0 ± 6·9**	136·2 ± 4·6**
PT-11	314·5 ± 4·8**	143·1 ± 3·6**

[a]Cortical cultures were treated with dammarane derivatives (1 μM) for one hour before glutamate-induced neurotoxicity. The values shown are the mean ± s.e.m. of three experiments. [b]Glutamate-treated value differs significantly from the untreated control ($P < 0{\cdot}001$). **$P < 0{\cdot}01$, compared with glutamate alone.

Discussion

We previously reported that ginsenosides Rb_1 and Rg_3 inhibited the over-production of NO and preserved the level of SOD in glutamate-treated cells. Furthermore, in cultures treated with glutamate, these ginsenosides inhibited the formation of malondialdehyde and diminished the influx of calcium. A drawback to these two natural compounds is that the ginsenosides must be present during the period of the neurotoxic challenge with glutamate to observe any significant ameliorative effect.

The results of this study demonstrate that chemically modified dammarane derivatives, MA-11 and PT-11, had a significant neuroprotective effect against glutamate-induced cortical neuronal damage in-vitro. They prevented a decrease in catalase activity and inhibited the formation of intracellular peroxide and malondialdehyde induced by the neurotoxicant. MA-11 and PT-11 could thus be useful neuroprotective agents that mitigate the oxidative stress caused by excess glutamate.

The neuroprotective activity of the PPT derivatives was less effective than that of PPD derivatives or ginsenosides Rb_1 and Rg_3 in preserving cell viability. However, all the PPT derivatives (except N-001) significantly reduced neuronal damage induced by glutamate. In our previous report, the neuroprotective effects of ginsenosides Rb_1 and Rg_3 began to decrease at 10 μM. At higher concentrations, the compounds appear to be cytotoxic to neuronal cells (Kim et al 1998). This phenomenon is also seen with saponin at concentrations above 10 μM (Hostettmann & Marston 1995). In this study, the neuroprotective activity of PPD and PPT, aglycones of dammarane derivatives, decreased significantly at concentrations above 10 μM, as with ginsenosides Rb_1 and Rg_3. However, the neuroprotective activity of four dammarane derivatives (MA-11, PT-11, POA-101 and POA-111) having aryl groups instead of hydroxyl groups at C-3, C-6 or C-12 was significantly maintained at a concentration of 10 μM. It might be caused by the loss of saponification by the masking original

Table 4. Effect of MA-11 and PT-11 on the activity of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase in glutamate-treated cortical cell cultures[a].

	SOD (mUnits mL^{-1})	Catalase (μmol H_2O_2 consumed min^{-1} (mg protein)$^{-1}$)	GSH-px (μmol NADPH consumed min^{-1} (mg protein)$^{-1}$)	GSSG-R (μmol NADPH consumed min^{-1} (mg protein)$^{-1}$)
Control	53·5 ± 4·4	38·1 ± 5·2	22·8 ± 2·7	12·1 ± 1·2
Glutamate[b]	22·6 ± 3·7	15·1 ± 3·2	7·2 ± 0·9	5·8 ± 3·2
MA-11	32·0 ± 2·6	27·8 ± 1·1**	8·2 ± 1·3	9·8 ± 0·5**
PT-11	29·8 ± 4·7	30·3 ± 2·1**	10·5 ± 2·7	10·3 ± 1·1**

[a]Cortical cultures were treated with dammarane derivatives (1 μM) for one hour before glutamate-induced neurotoxicity. The cultures were then exposed to 50 μM glutamate. After 24 h incubation in DMEM in the presence of dammarane derivatives, the cultures were assessed for the extent of neuronal damage. The values shown are the mean ± s.e.m. of three experiments. [b]Glutamate-treated value differs significantly from the untreated control ($P < 0{\cdot}001$). **$P < 0{\cdot}01$, compared with glutamate alone.

hydroxyl groups in authentic ginsenosides. These results suggested that our semi-synthetic dammarane derivatives are less cytotoxic on glutamate-insulted cultures than ginsenosides.

It is well known that Ca^{2+} mediates a delayed neuronal degeneration caused by the activation of NMDA and non-NMDA receptors (Coyle & Puttfarcken 1993). The Ca^{2+} influx brought about by glutamate receptor activation causes oxidative stress that leads to neuronal degeneration. MA-11 and PT-11 had little effect in reducing Ca^{2+} entry caused by excess glutamate (Table 3). Treatment with MA-11 and PT-11 had less effect than that with ginsenosides Rb_1 and Rg_3 on Ca^{2+} entry. This result suggests that MA-11 and PT-11 might not directly act on glutamatergic receptors or Ca^{2+} channels. However, further experiments will be carried out to confirm this proposed mechanism.

Hydrogen peroxide is produced from superoxide anion by SOD and is then cleared to water by catalase (Olanow 1993). The reduction of molecular oxygen to water in glutamate toxicity involves the formation of the most potent oxidant, the hydroxyl radical (Yu 1994). The preservation of catalase by MA-11 and PT-11 increased the clearance of hydrogen peroxide and hydroxyl radicals, subsequently reducing oxidative stress. Glutathione (GSH) is the substrate in the GSH-redox cycle and also reacts directly with superoxide radicals, hydroxy radicals and organic free radicals (Sampath et al 1994; Yu 1994). GSSG-R is involved in maintaining cellular homeostasis of reduced GSH, together with GSH-px. Therefore, preserved activity of GSSG-R by MA-11 and PT-11 may increase the content of reduced GSH and be involved in the clearance of free radicals together with catalase. Indeed, the reduction of intracellular peroxide was observed in the glutamate-treated cells by the preservations of catalase and GSSG-R activity.

Furthermore, MA-11 and PT-11 inhibited the formation of malondialdehyde in cortical cell cultures exposed to glutamate, suggesting that lipid peroxidation is reduced as the result of a decrease in hydroxyl radicals and hydrogen peroxide. Therefore, we postulated that MA-11 and PT-11 might affect inhibition of Ca^{2+}-dependent enzyme over-activation rather than the inhibition of excess Ca^{2+} influx.

At present, the cellular and molecular mechanisms that underlie the action of MA-11 and PT-11 are not fully understood. However, these results suggested that MA-11 and PT-11 had significant neuroprotective activity and had less cytotoxic activity than ginsenosides Rb_1 and Rg_3.

Although dammarane derivatives, MA-11 and PT-11, had significant neuroprotective activity without potentiating neuronal damage at concentrations over 10 μM, this neuroprotective activity was not superior to that of ginsenosides Rb_1 and Rg_3.

Further studies for seeking dammarane derivatives which could be useful neuroprotective agents are needed.

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