Arch Pharm Res Vol 25, No 3, 349-356, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Protective Effect of Fangchinoline on Cyanide-Induced Neurotoxicity in Cultured Rat Cerebellar Granule Cells

Soon Ok Cho and Yeon Hee Seong

College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, 361-763, Korea

(Recevied February 26, 2002)

The present study was performed to examine the effect of fangchinoline, a *bis*- benzylisoquinoline alkaloid, which exhibits the characteristics of a Ca^{2+} channel blocker, on cyanide-induced neurotoxicity using cultured rat cerebellar granule neurons. NaCN produced a concentration-dependent reduction of cell viability, which was blocked by MK-801, an N-methyl-D-aspartate (NMDA) receptor antagonist, verapamil, L-type Ca^{2+} channel blocker, and L-NAME, a nitric oxide synthase inhibitor. Pretreatment with fangchinoline over a concentration range of 0.1 to 10 μM significantly decreased the NaCN-induced neuronal cell death, glutamate release into medium, and elevation of $[Ca^{2+}]_i$ and oxidants generation. These results suggest that fangchinoline may mitigate the harmful effects of cyanide-induced neuronal cell death by interfering with $[Ca^{2+}]_i$ influx, due to its function as a Ca^{2+} channel blocker, and then by inhibiting glutamate release and oxidants generation.

Key words: Fangchinoline, NaCN, Neurotoxicity, Ca^{2+} channel antagonist, Cerebellar granule cells

INTRODUCTION

Cyanide is one of the most rapid-action poisons available against mammals. The central nervous system (CNS), including respiratory distress, seizures and convulsions, is a primary target organ in cyanide toxicity (Way, 1984). In some individuals, a Parkinsonism-like condition may develop as a post-toxicity sequela (Uitti *et al.*, 1985). Cyanide exerts its toxic action by interfering with cellular respiration through the inhibition of cytochrome oxidase. Therefore, cyanide poisoning has been frequently used as a model of chemical hypoxia. Excitatory amino acids such as glutamate and aspartate, which are important and abundant neurotransmitters in CNS, play an important part in cyanide neurotoxicity. Cyanide induces the release of glutamate from neuronal stores and alters its brain levels (Patel *et al.*, 1991). The consequently increased extracellular levels of glutamate may result in overstimulation of glutamate receptors, leading to excitotoxic responses (Rothman, 1984). N-methyl-D-aspartate (NMDA) receptor-mediated Ca^{2+} influx appears to be a key in the neurotoxic process initiated by cyanide (Sun *et al.*, 1997). Therefore, in neuronal cells, specific-NMDA receptor antagonists such as APV and MK-801 block cyanide-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) elevation and prevent neuronal cytotoxicity (Cai and McCaslin, 1992; Pauwels *et al.*, 1989). Characterization of a chemical anoxia model in cerebellar granule neurons using sodium azide was protected by nifedipine, a blocker of L-type voltage-sensitive Ca^{2+} channels, and MK-801 (Varming *et al.*, 1996). Oxidative stress also plays a role in cyanide neurotoxicity (Ardelt, 1989). Johnson *et al.* (1987) proposed that increased $[Ca^{2+}]_i$ after KCN treatment generates reactive oxygen species (ROS) ($O_2^{\cdot-}$, $^{\cdot}OH$) and nitric oxide (NO) leading to lipid peroxidation and neuronal damage. Primary cultured cerebellar granule cells have been used extensively to study the mechanisms of neuronal death. This is in part due to the fact that these are predominantly glutamatergic neurons, and glutamate-receptor-mediated excitotoxicity is believed to play a role in the pathophysiology of neurodegenerative diseases (Manev *et al.*, 1990).

Fangchinoline (6,6',12-trimethoxy-2,2'-dimethyl-berbaman-7-ol), together with its 7-methylated derivative, tetrandrine, is a *bis*-benzylisoquinoline alkaloid derived from the Chinese

Correspondence to: Yeon Hee Seong, College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, 361-763, Korea
E-mail : vepharm@chungbuk.ac.kr

herb *Radix Stephania tetrandra.* These *bis*-benzylisoquinoline alkaloids have been characterized pharmacologically to exhibit Ca^{2+} antagonistic, hypotensive, immunosuppressive and anti-inflammatory properties, etc (Ivanovska *et al.*, 1999a and 1999b; Jiangsu College of New Medicine, 1975; Kawashima *et al.*, 1990; Liu *et al.*, 1995). Recent studies have shown that fangchinoline inhibits histamine release, platelet aggregation, platelet thromboxane formation, and dopamine biosynthesis in *in vitro* assays, and that it lowers blood pressure as a non-specific Ca^{2+} channel antagonist (Kim *et al.*, 1997, 1998 and 1999; Nakamura *et al.*, 1992).

In a previous report, we demonstrated that fangchinoline prevented excitatory amino acids-induced neurotoxicity, via inhibition of Ca^{2+} influx (Kim *et al.*, 2001). Thus, in the present study, the effect of fangchinoline on NaCN-induced neuronal cell death was studied in cultured rat cerebellar granule cells. Furthermore, in order to investigate the mechanism involved, the effects of fangchinoline on NaCN-induced glutamate release, $[Ca^{2+}]_i$ elevation and oxidants generation were studied.

MATERIALS AND METHODS

Materials

Fangchinoline was isolated from the creeper *Stephania tetrandra* S. Moore (or *fenfangji*), and confirmed by comparing its physical and chemical properties, and ^{1}H-NMR spectra, with those of previous reports (Lin *et al.*, 1993). Dulbecco's modified Eagle's medium (DMEM), poly-L-lysine, NaCN, (±)verapamil and cytosine arabinoside were purchased from Sigma Chemical Co. (St. Louis, MO, USA), MK-801 and L-NAME from Research Biochemicals International (Natick, MA, USA), fluo-3/acetoxymethyl ester (Fluo-3, AM) and 2, 7-dichlorofluorescin diacetate from Molecular probes (Eugene, OR, USA), and fetal bovine serum from Hyclone (Logan, Utah, USA). All other chemicals used were of the highest grade available.

Primary culture of cerebellar granule neurons

Cerebellar granule cells were cultured as described previously (Seong *et al.*, 2000). Briefly, 7 to 8-day-old rat pups (Sprague-Dawley) were decapitated, and the heads were partially sterilized by dipping in 95% ethanol. The cerebellum was dissected from the tissue and placed in medium lacking serum and bicarbonate, but containing trypsin (0.25 mg/ml). Cells dissociated by trypsinization and slight trituration were collected by centrifugation and resuspended in DMEM supplemented with sodium pyruvate (0.9 mM), glutamine (3.64 mM), sodium bicarbonate (40 mM), glucose (22.73 mM) and 10 % fetal bovine serum. Cells were seeded at a density of about 2×10^6 cells/ml into poly-L-lysine coated 12 well-plates or glass coverslips for measurement of $[Ca^{2+}]_i$ and oxidants. After 2 days incubation, growth medium was aspirated from the cultures and new growth medium containing 25 mM KCl and 20 uM cytosine arabinoside, to prevent proliferation of nonneuronal cells, was added. Cultures were kept at 37°C in a 7% CO_2 atmosphere.

Neurotoxicity experiments

Neurotoxicity experiments were performed on neurons grown for 8-10 days *in vitro* on either 12-well culture plates or glass coverslips placed in culture dishes. The culture medium was removed and neurons were washed with a HEPES-buffered solution (incubation buffer) containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, and 1 mM $MgCl_2$, at pH 7.4. They were then incubated for 30 min in the same medium, and further incubated for 60 min (unless otherwise indicated) in the presence of NaCN at 37°C. To confirm the chemical hypoxia of cells, glucose was omitted from the incubation buffer. Fangchinoline and other inhibitors were added to the medium 15 min prior to the treatment with NaCN.

Assay of cell viability (trypan blue exclusion assay)

Upon completion of incubation, cells were stained with 0.4 % trypan blue solution at room temperature for 10 min. Only dead cells with a damaged cell membrane are permeable to trypan blue. The numbers of trypan blue-permeable blue cells and viable white cells were counted in six randomly chosen fields or culture wells. Experiments were performed in triplicate with at least 3 different batches.

Assay of mitochondrial activity (MTT assay)

Cell survival was also measured by the level of mitochondrial respiration in cells after NaCN treatment, as measured by the reduction of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), into a blue formazan precipitate. After NaCN treatment, cells were incubated in serum-free growth medium containing MTT (0.5 mg/ml) for 4 hr at 37°C. The blue formazan produced was solubilized in 0.4 ml of acid-isopropanol (0.04 N HCl in isopropanol), and the optical density was read at 570 nm. Only cells with functional mitochondria are capable of cleaving MTT to generate the dark purple formazan. Results were expressed as percentage of control (Mosmann *et al.*, 1983).

Measurement of glutamate concentration

After treatment of cells, a sample was taken from the buffer for determination of the level of glutamate secreted

into the buffer. Glutamate was quantified by high performance liquid chromatography with an electrochemical detector (Ellison *et al.*, 1987). Briefly, after a small aliquot was collected from the culture wells, glutamate was separated on an analytical column (ODS2; particle size, 5 μm; 4.6 × 100 mm) after pre-derivatization with O-phthaldialdehyde/2-mercaptoethanol. Derivatives were detected by electrochemistry at 0.1 μA/V, and the reference electrode was set at 0.7 V. The column was eluted with mobile phase (pH 5.20) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a flow rate of 0.5 ml/min.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured using a laser scanning confocal microscope with the Ca^{2+} indicator, fluo-3, AM, as described by Lee *et al.* (1998). Cells, grown on coverslips and serum-starved for 1 hr, were incubated with 5 μ M fluo-3, AM in serum-free DMEM for 40 min and washed with the incubation buffer. Each coverslip containing fluo-3, AM labeled cells was mounted on a perfusion chamber, subjected to confocal laser scanning microscopy (Carl Zeiss LSM 410), and then scanned every second with a 488 nm excitation argon laser and a 515 nm longpass emission filter. NaCN was added to the cells by using an automatic pumping system. In order to test the effect of fangchinoline or MK-801 on NaCN-induced $[Ca^{2+}]_i$ change, cells were pretreated with the compounds for 15 min and challenged by the compounds during the incubation with NaCN. All scanning images were processed to analyze changes of $[Ca^{2+}]_i$ at a single cell level. The results were expressed as the relative fluorescence intensity (Lee et al., 1998).

Measurement of oxidants generation

The microfluorescence assay of 2, 7-dichlorofluorescin, the fluorescent product of 2, 7-dichlorofluorescin diacetate (DCF-DA), was used to monitor generation of oxidants, ROS and NO (Gunasekar, 1995). Oxidants generation was monitored by a laser scanning confocal microscope (Carl Zeiss LSM 410). Cells, grown on coverslips, were washed with DMEM (phenol red-free) three times and incubated with the buffer for 30 min at 37°C. Then, the buffer was changed for the incubation buffer containing 10 mM NaCN, and cells were further incubated for 30, 60 or 90 min. The uptake of DCF-DA (5 uM) dissolved in dimethylsulfoxide was carried out for the last 10 min of the incubation with NaCN. Nest, coverslips containing granule cells loaded with DCF-DA were mounted on the confocal microscope stage after washing with buffer. Immediately after mounting, the intensity of fluorescence induced by generated oxidants was measured. The fluorescent intensity induced by DCF-DA loaded cells without any treatment was regarded as the control level.

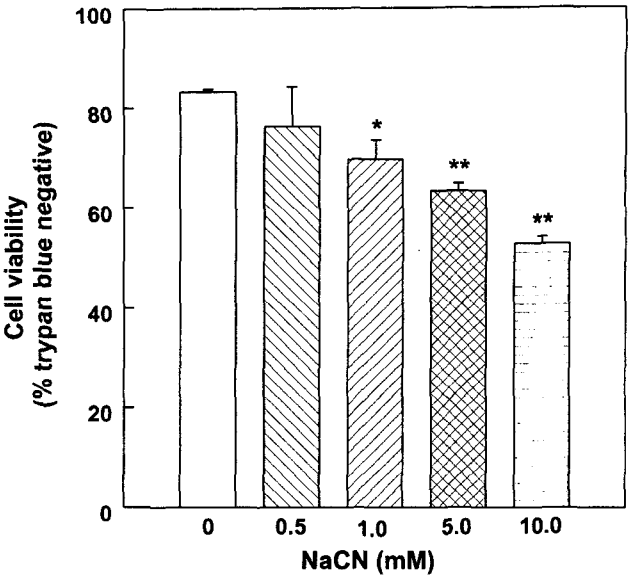

Fig. 1. Concentration-response of NaCN on the cell viability of cultured cerebellar granule neurons. Cultured cells were incubated with various concentrations of NaCN (1-10 mM) at 37°C for 60 min. After completion of incubation, trypan blue exclusion test was performed. Values represent the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, compared to control.

Statistical Analysis

Data were expressed as meanSEM and statistical significance was assessed by one-way analysis of variance (ANOVA) followed by the Student's *t*-test in all experiments.

RESULTS

Inhibitory effect of fangchinoline on NaCN-induced neuronal cell death

Cell death after plasma membrane damage was assayed by the ability of cerebellar granule neurons to take up trypan blue. Cell exposure to NaCN (≥1 mM) for 1 hour increased the number of cells stained by trypan blue (Fig. 1). The morphological changes of cerebellar granule neurons after exposure to NaCN were characterized by decomposition of neuronal aggregates, fragmentation of neurite and loss of soma. As shown in Fig. 2, exposure to NaCN (10 mM) produced significant neuronal cell death, with cell viability decreasing from 75.5% in the control cells to 43.0% in the NaCN-exposed cells. Fangchinoline (0.1, 1 and 10 μM) produced a significant inhibitory effect on NaCN (10 mM)-induced neuronal cell death, resulting in 62.0 % cell viability at the concentration of 10 μM. Fangchinoline (10 μM) alone, did not affect the cell

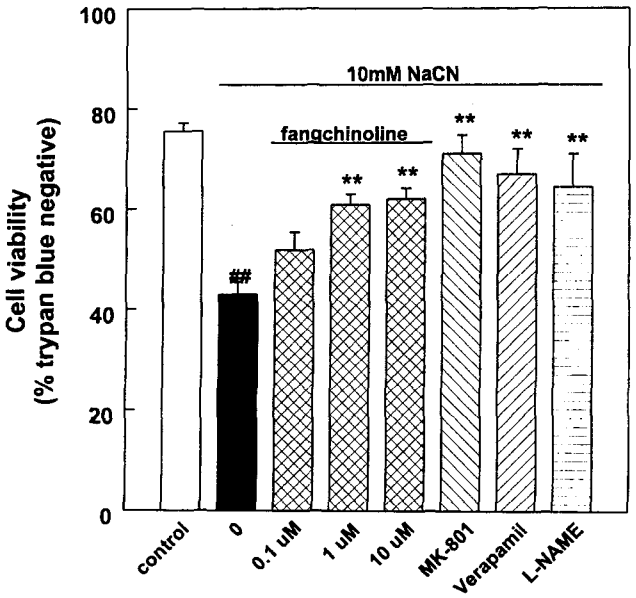

Fig. 2. Effects of fangchinoline, verapamil, MK-801 and L-NAME on NaCN-induced cell death in cerebellar granule neurons. Cells were incubated with NaCN (10 mM) at 37°C for 60 min in the presence or absence of fangchinoline, MK-801 (10 μM), verapamil (10 μM) or L-NAME (1 mM). The inhibitors were added to cells 15 min prior to NaCN treatment. After completion of incubation, trypan blue exclusion test was performed. Values represent the mean ± SEM. ## $p<0.01$, compared to control. ** $p<0.01$, compared to 10 mM NaCN.

viability (data not shown). MK-801 (10 μM), verapamil (10 μM), and L-NAME (1 mM) also inhibited NaCN-induced cell death, exhibiting 71%, 66.9% and 64.4% cell viability, respectively.

To further assess NaCN-induced neuronal cell death, MTT assay was performed. Normally, mitochondrial enzymes are capable of transforming MTT tetrazolium salt into MTT formazan. Thus, MTT assay is extensively used as a sensitive, quantitative and reliable colorimetric assay for cell viability. The capacity of MTT reduction of cells was decreased to 84.8% by 10 mM NaCN, relative to control MTT reduction. Fangchinoline (0.1, 1 and 10 μM) inhibited this NaCN-induced decrease of MTT reduction capacity, showing 98.6% of control MTT reduction at 10 μM. Similarly, MK-801 (10 μM) and verapamil (10 μM) significantly reduced this MTT reduction decrease (105.9% and 93.8% of control MTT reduction, respectively) (Fig. 3).

Inhibitory effect of fangchinoline on NaCN-induced elevation of glutamate release

Glutamate release, as an indicator of cyanide-induced neurotoxicity, was measured after the incubation of cells with NaCN for 1 hr. As shown in Fig. 4, 10 mM NaCN

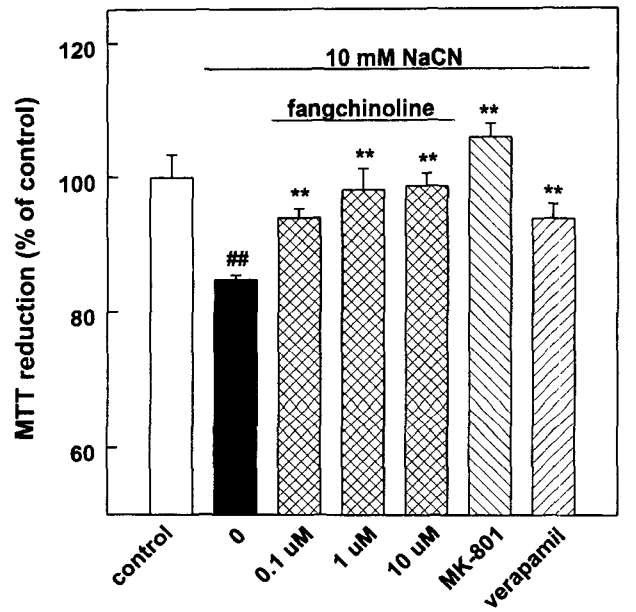

Fig. 3. Effects of fangchinoline, MK-801 and verapamil on NaCN-induced decrease of MTT reduction in cerebellar granule neurons. Cells were incubated with NaCN (10 mM) at 37°C for 60 min in the presence or absence of fangchinoline, MK-801 (10 μM) or verapamil (10 M). The inhibitors were added to cells 15 min prior to NaCN treatment. After completion of incubation, cells were processed for MTT assay. Values represent the mean ± SEM. ## $p<0.01$, compared to control. * $p<0.05$, ** $p<0.01$, compared to 10 mM NaCN.

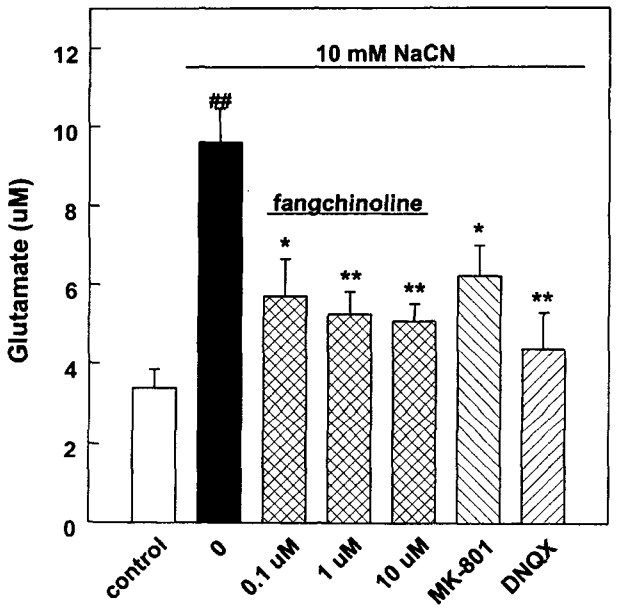

Fig. 4. Effects of fangchinoline, MK-801 and DNQX on NaCN-induced glutamate release. Cultured cells were incubated with NaCN (10 mM) at 37°C for 60 min in the presence or absence of fangchinoline, MK-801 (10 μM) or DNQX (20 μM). The inhibitors were added to cells 15 min prior to NaCN treatment. After completion of incubation, the amount of glutamate secreted into the buffer was measured by HPLC with ECD. Values represent the mean ± SEM. ## $p<0.01$, compared to control. * $p<0.05$, ** $p<0.01$, compared to 10 mM NaCN.

markedly elevated the basal glutamate level from 3.4 to 9.6 μM, while fangchinoline (0.1, 1 and 10 μM) significantly blocked the NaCN-induced elevation of glutamate release, as did MK-801 (10 μM), from 9.6 μM to 6.2 μM, and DNQX (10 μM), a non-NMDA glutamate receptor antagonist. These results suggest that NaCN-induced neurotoxicity is related to not only NMDA receptor but also non-NMDA receptors.

Inhibitory effect of fangchinoline on NaCN-induced elevation of $[Ca^{2+}]_i$

The elevation of $[Ca^{2+}]_i$ by NaCN was measured by laser scanning confocal microscopy after loading fluorescent dye, fluo-3, AM, to the cells. In cerebellar granule neurons, treatment with NaCN (1 mM) induced very slow and gradual increase of $[Ca^{2+}]_i$, beginning at 4-5 min after NaCN application. A maximal fluorescent intensity of 150 (compared to a base of 25) induced by $[Ca^{2+}]_i$ elevation was measured 15-18 min after the NaCN application. After peaking, the fluorescence level was decreased gradually. On the other hand, NaCN application in the presence of fangchinoline (5 μM) or MK-801 (10 μM) failed to induce the elevation of $[Ca^{2+}]_i$ throughout the period of measurement (Fig. 5).

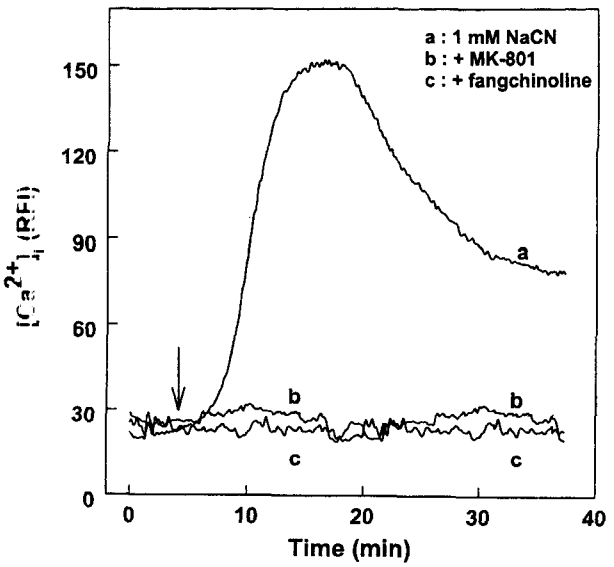

Fig. 5. Effects of fangchinoline and MK-801 on NaCN-induced elevation of $[Ca^{2+}]_i$. After loading with 5 μM fluo-3, AM for 40 min, cells on coverslips were mounted on a perfusion chamber, subjected to confocal laser scanning microscopy to monitor $[Ca^{2+}]_i$ change, and perfused with incubation buffer. Fangchinoline (5 μM) or MK-801 (10 μM) was added to cells 15 min prior to NaCN treatment, before commencement of $[Ca^{2+}]_i$ monitoring. Results are expressed as the relative fluorescence intensity (RFI). Each trace is a single cell representative. The arrow shows the time of NaCN addition.

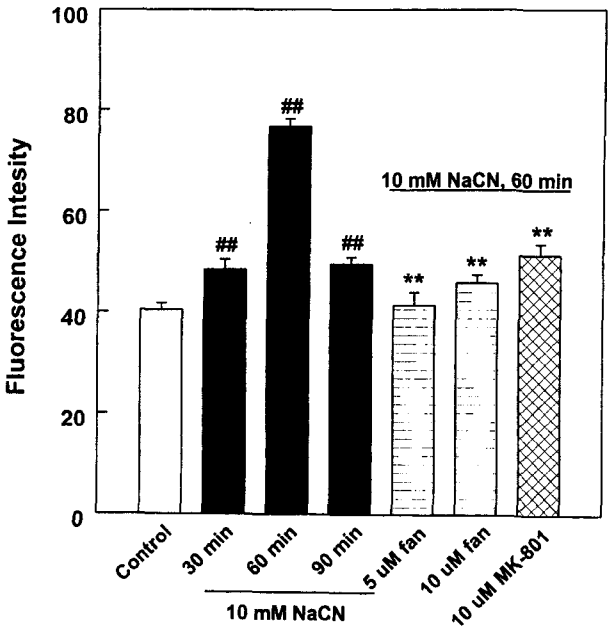

Fig. 6. Effects of fangchinoline and MK-801 on NaCN induced generation of oxidants in cerebellar granule neurons. Cells were incubated with NaCN at 37°C for 30, 60 or 90 min, in the presence or absence of fangchinoline or MK-801 in the incubation buffer. Fangchinoline or MK-801 was added to cells 15 min prior to NaCN treatment. Oxidants were monitored using laser scanning confocal microscopy by measurement of fluorescence intensity after loading DCF-DA during the last 10 min of the NaCN incubation. Values represent the mean ± SEM of RFI. ## $p<0.01$, compared to control. ** $p<0.01$, compared to 10 mM NaCN, 60 min.

Inhibitory effect of fangchinoline on NaCN-induced oxidants generation

NaCN-induced glutamate release and a cytosolic high concentration of free Ca^{2+}. Furthermore, the pathological condition induced by cyanide is associated with accelerated formation of ROS and NO. In DCF-DA-loaded cerebellar granule cells, NaCN increased the fluorescent intensity, indicating the generation of oxidants. It was demonstrated that the generation of oxidants by NaCN was maximal at 60 min, being about double the level of the control cells. The increase of fluorescent intensity produced by NaCN (10 mM) was significantly blocked by fangchinoline (5 and 10 μM) and MK-801 (10 μM) (Fig. 6).

DISCUSSION

Cyanide-induced neurotoxicity is associated with glutamate release from intracellular stores. Cyanide initiates an excitotoxic-like reaction in cerebellar granule cells which is primarily mediated by activation of the NMDA receptor (Gunasekar *et al.*, 1995 and 1996). This

activation promotes the influx of cations, including Na^+ and Ca^{2+}, which can lead to membrane depolarization. In turn, depolarization can activate membrane voltage-sensitive Ca^{2+} channels leading to additional Ca^{2+} influx. This influx stimulates concurrent generation of NO and ROS which then results in lipid peroxidation and cellular injury (Gunasekar et al, 1996 and 1998). The resultant neuronal damage therefore causes irreversible physiological disorder. The present study confirmed the previous observations (Ardelt *et al.*, 1989; Cai and McCaslin, 1992; Patel *et al.*, 1991; Pauwels *et al.*, 1989) that cyanide in cerebellar granule cells stimulated glutamate release, elevation of $[Ca^{2+}]_i$ and oxidants generation, which in turn led to neuronal cell death.

Fangchinoline is known to inhibit histamine release in an *in vitro* assay (Nakamura *et al.*, 1992) and to lower blood pressure as a non-specific Ca^{2+} channel antagonist (Kim et al, 1997). The effect of Ca^{2+} channel blockers on glutamate-induced neurotoxicity has been confirmed by many studies (Freund and Reddig, 1994; Weiss *et al.*, 1990). The present study was designed to investigate the inhibition of fangchinoline, and its mechanism, on cyanide-induced neurotoxicity. NaCN caused neuronal cell death, which was blocked by treatment with MK-801, verapamil and L-NAME. This result implies the involvement of NMDA-glutamate receptor activation, increase of Ca^{2+} influx and NO generation in cyanide-induced neurotoxicity in cultured cerebellar granule neurons, as has been previously evidenced in other studies.

Most of the previous hypotheses dealing with neurodegenerative diseases have invoked abnormal release and/or decreased uptake of glutamate as playing a key role in the process of excitotoxicity, for example, the increased glutamate release by ischemic and insulin-induced hypoglycemia as proposed by Coyle and Puttfarcken (1993). The release of endogenous glutamate, which acts on glutamate receptors, secondly triggers Na^+ influx and neuronal depolarization. This leads to Cl^- influx down its electrochemical gradient, further cationic influx and osmotic lysis of the neuron, resulting in neuronal cell death (Van Vliet *et al.*, 1989). According to many reports, when cerebellar granule neurons were exposed to NaCN, extracellular glutamate level and $[Ca^{2+}]_i$ were significantly increased. The NaCN-induced elevation of glutamate release was significantly inhibited by MK-801 and DNQX, implying that cyanide-induced neurotoxicity was involved in the activation of not only NMDA, but also non-NMDA glutamate receptors. Fangchinoline over the concentration range of 0.1 to 10 μM also significantly inhibited the NaCN-induced glutamate elevation. A sustained increase in $[Ca^{2+}]_i$ triggers a series of events including cGMP level elevation, glutamate release and NOS activation (Baltrons *et al.*, 1997; Mei *et al.*, 1996). Released glutamate secondly acts on glutamate receptors and therefore potentiates the neurotoxicity. This condition is reversed by using Ca^{2+} channel antagonists and MK-801. In the present experiments, the NaCN-induced elevation of $[Ca^{2+}]_i$ was markedly inhibited by MK-801 and fangchinoline. Furthermore, fangchinoline, at the concentrations of 5 and 10 μM, completely inhibited the NaCN-induced generation of oxidants. These results suggest that fangchinoline prevented cyanide-induced neurotoxicity via Ca^{2+}-channel antagonistic activity. *Bis*-benzylisoquinoline alkaloids have been traditionally used in the clinical treatment of high blood pressure and tumors in China. As for the effects of these compounds on lowering blood pressure, tetrandrine was reported to inhibit the voltage-dependent Ca^{2+} channels (Felix *et al.*, 1992; Liu *et al.*, 1995), and to act as a receptor-operated Ca^{2+} channel blocker and/or an inhibitor of Ca^{2+} release from intracellular pools (Leung *et al.*, 1994; Takemura *et al.* 1995). Fangchinoline is also known to demonstrate the vasodilation and inhibition of high K^+- and NE-induced Ca^{2+} influx in rat aorta strips (Kim *et al.* 1997). Tetrandrine exhibited a protective effect against ischemia-reperfusion brain damage in gerbils (Sun and Liu; 1995). We also demonstrated that fangchinoline prevented excitatory amino acids-induced neurotoxicity in a previous report (Kim *et al.*, 2001). In line with these reports, the present study strongly suggests that fangchinoline might be of value in preventing the ischemic neurodegenerative pathophysiological condition.

In summary, fangchinoline, which mainly acts on Ca^{2+} channels, effectively prevented NaCN-induced neuronal cell death in cultured cerebellar granule neurons, via the inhibition of Ca^{2+} entry, of related glutamate release caused by glutamate receptors activation, and of oxidants generation.

REFERENCES

Ardelt, B. K., Borowitz, J. L. and Isom, G. E., Brain lipid peroxidation and antioxidant protectant mechanisms following acute cyanide intoxication. *Toxicol.*, 56, 147-154 (1989).

Baltrons, M. A., Saadoun, S., Agullo, L. and Garcia, A., Regulation by calcium of the nitric oxide/cyclic GMP system in cerebellar granule cells and astroglia in culture. *J. Neurosci. Res.*, 49(3), 333-341 (1997).

Cai, Z. and McCaslin, P. P., Selective effects of cyanide (100 μM) on the excitatory amino acid-induced elevation of intracellular calcium levels in neuronal culture. *Neurochem. Res.*, 17, 803-808 (1992).

Coyle, J. T. and Puttfarcken, P., Oxidative stress, glutamate and neurodegenerative disorders. *Science*, 262(29), 689-694 (1993).

Ellison, D. W., Beal, M. F. and Martin, J. B., Amino acid neurotransmitters in postmortem human brain analyzed by

high performance liquid chromatography with electrochemical detection. *J. Neurosci.,* 19, 305-315 (1987).

Felix, J. P., King, V. F., Shevell, J. L., Garcia, M. L., Kaczorowski, G. J., Bick, R. and Slaughter, R. S., Bis(benzylisoquinoline) analogs of tetrandrine block L-type calcium channels: evidence for interaction at the diltiazem-binding site. *Biochem.,* 31(47), 11793-11800 (1992).

Freund, W. and Reddig, S., AMPA/Zn^{2+}-induced neurotoxicity in rat primary cortical cultures: involvement of L-type calcium channels. *Brain Res.*, 654, 257-264 (1994).

Gunasekar, P. G., Borowitz, J. L. and Isom, G. E., Cyanide-induced generation of oxidative species : involvement of nitric oxide synthase and cyclooxygenase-2. *J. Pharmacol. Exp. Ther.,* 285(1), 236-241 (1998).

Gunasekar, P. G., Kanthasamy, A. G., Borowitz, J. L. and Isom, G. E., Monitoring intracellular nitric oxide formation by dichlorofluorescin in neuronal cells. *J. Neurosci. Meth.,* 61, 15-21 (1995).

Gunasekar, P. G., Kanthasamy, A. G., Borowitz, J. L. and Isom, G. E., NMDA receptor activation produces concurrent generation of nitric oxide, a reactive oxygen species: implication for cell death. *J. Neurochem.,* 65(5), 2016-2021 (1995).

Gunasekar, P. G., Sun, P. W., Kanthasamy, A. G., Borowitz, J. L. and Isom, G. E., Cyanide-induced neurotoxicity involves nitric oxide and reactive oxygen species generation after N-Methyl-D-aspartate receptor activation. *J. Pharmacol. Exp. Ther.,* 277, 150-155 (1996).

Ivanovska, N., Hristova, M. and Philipov, S., Complement modulatory activity of bisbenzylisoquinoline alkaloids isolated from isopyrum thalictroides--II. Influence on C3-9 reactions vitro and antiinflammatory effect in vivo. *Int. J. Immunopharmacol.*, 21(5), 337-47 (1999b).

Ivanovska, N., Nikolova, P., Hristova, M., Philipov, S. and Istatkova, R., Complement modulatory activity of bisbenzylisoquinoline alkaloids isolated from isopyrum thalictroides--I. Influence on classical pathway in human serum. *Int. J. Immunopharmacol.*, 21(5), 325-36 (1999a).

Jiangsu college of New Medicine, *Encyclopedia of Material Medica*, vol. 1. Shanghai People's publishing House, Shanghai, China, pp. 981-985 (1975).

Johnson, J. D., Conroy, W. G., Burris, K. D. and Isom, G. E., Peroxidation of brain lipids following cyanide intoxication in mice. *Toxicology,* 46(1), 21-28 (1987).

Kawashima, K., Hayakawa, T., Miwa, Y., Oohata, H., Suzuki, T., Fujimoto, K., Ogino, T. and Chen, Z. X., Structure and hypotensive activity relationships of tetrandrine derivatives in stroke-prone spontaneously hypertensive rats. *Gen. Pharmacol.,* 21, 343-347 (1990).

Kim H. S., Zhang, Y. H., Fang, L. H., Yun, Y. P. and Lee, M. K., Effects of tetrandrine and fangchinoline on human platelet aggregation and thromboxane B2 formation. *J. Ethnopharmacol.*, 66(2), 241-246 (1999).

Kim, H. S., Zhang, Y. H., Lee, M. K. and Fang, L. H., Inhibitory effects of fangchinoline on dopamine biosynthesis in PC12 cells. *Med. Sci. Res.*, 26, 475-476 (1998).

Kim, H. S., Zhang, Y. H., Oh, K. W. and Ahn, H. Y., Vasodilating and hypotensive effects of fangchinoline and tetrandrine on the rat aorta and the stroke-prone spontaneously hypertensive rat. *J. Ethnopharmacol.*, 58, 117-123 (1997).

Kim, S. D., Oh, S. K., Kim, H. S. and Seong, Y. H., Inhibitory effect of fangchinoline on excitatory amino acids-induced neurotoxicity in cultured rat cerebellar granule cells. *Arch. Pharm. Res.*, 24(2), 164-170 (2001).

Lee, Z. W., Kweon, S. M., Kim, B. C., Leem, S. H., Shin, I. C., Kim, J. H. and Ha, K. S., Phosphatidic acid-induced elevation of intracellular Ca^{2+} is mediated by RhoA and H_2O_2 in rat-2 fibroblasts. *J. Biol. chem.,* 22, 12710-12715 (1998).

Leung, Y. M., Kwan, C. Y. and Loh, T. T., Dual effects of tetrandrine on cytosolic calcium in human leukaemic HL-60 cells: intracellular calcium release and calcium entry blockade. *Br. J. Pharmacol.,* 113, 767-774 (1994).

Lin, L. Z., Shieh, H. L., Angerhofer, C. K., Pezzuto, J. M., Cordell, G. A., Xue, L., Johnson, M. E. and Ruangrungsi, N., Cytotoxic and antimalarial bisbenzylisoquinoline alkaloids from cyclea barbata. *J. Nat. Prod.*, 56, 22-29 (1993).

Liu, Q. Y., Li, B., Gang, J. M., Karpinski, E. and Pang, P. K., Tetrandrine, a Ca^{2+} antagonist: effects and mechanisms of action in vascular smooth muscle cells. *J. Pharmacol. Exp. Ther.,* 273, 32-39 (1995).

Manev, H., Costa, E., Wroblewski, J. T. and Guidotti, A., Abusive stimulation of excitatory amino acid receptors: a strategy to limit neurotoxicity. [Review] [46 refs] *FASEB J.* 4(10), 2789-2797 (1990).

Mei, J. M., Chi, W. M., Trump, B. F. and Eccles, C. U., Involvement of nitric oxide in the degeneration of cytosolic calcium in cerebellar neurons during combined glucose-oxygen deprivation. *Mol. Chem. Neuropathol.,* 27(2), 155-166 (1996).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods,* 65(1-2), 55-63 (1983).

Nakamura, K., Tsuchiya, S. and Sugimoto, Y., Histamine release inhibition activity of bis-benzyl-isoquinoline alkaloids. *Planta Med.,* 58, 505-508 (1992).

Patel, M. N., Ardelt, B. K., Yim, G. K. W. and Isom, G. E., Cyanide induces Ca^{2+} dependent and independent release of glutamate from mouse brain slices. *Neurosci. Lett.,* 131, 42-44 (1991).

Pauwels, P. J., Van Assouw, H. P., Leysen, J. E. and Janssen, P. A. J., Ca^{2+}-mediated neuronal death in rat brain neuronal cultures by veratridine : protection by flunarizine. *Mol. Pharmacol.,* 36, 525-531 (1989).

Rothman, S. M., Synaptic release of excitatory amino acid neurotransmitter mediates anoxic neuronal death. *J. Neurosci.,* 4, 1884-1891 (1984).

Seong, Y. H., Koh, S. B. and Jo, S. O., Protective effects of ginsenosides on cyanide-induced neurotoxicity in cultured rat cerebellar granule cells. *J. Ginseng Res.*, 24(4), 196-201 (2000).

Sun, F. and Liu, T. P., Tetrandrine vs. nicardipine in cerebral ischemia-reperfusion damages in gerbils. *Chung-Kuo Yao Li Hsueh Pao-Acta Pharmacologica Sinica.* 16(2), 145-148 (1995).

Sun, P. W., Rane, S. G., Gunasekar, P. G., Borowitz, J. L. and Isom, G. E., Modulation of the NMDA receptor by cyanide : Enhancement of receptor-mediated responses. *J. Pharmacol. Exp. Ther.,* 280, 1341-1348 (1997).

Takemura, H., Kwan, C. Y. and Ohshika, H., Calcium antagonistic actions of tetrandrine depend on cell types. *Res. Com. Mol. Pathol.. & Pharmacol.,* 90(1), 59-68 (1995).

Uitti, R. J., Rajput, A. H., Ashenhurst, E. M. and Rozdilsky, B., Cyanide-induced parkinsonism. *A Clin. Pathol. Rep. Neurol.,* 35, 921-925 (1985).

Van Vliet, B. J., Sebben, M., Dumuis, A., Gabrion, J., Bockaert, J. and Pin, J. P., Endogenous amino acid release from cultured cerebellar neuronal cells: Effect of tetanus toxin on glutamate release. *J. Neurochem.*, 52(4), 1229-1230 (1989).

Varming, T., DreJer, J., Frandsen, A. and Schousgoe, A., Characterization of a chemical anoxia model in cerebellar granule neurons using sodium azide : protection by nifedipine and MK-801. *J. Neurosci. Res.,* 44, 40-46 (1996).

Way, J. L., Cyanide intoxication and its mechanism of antagonism. *Annu. Rev. Pharmacol. Toxicol.,* 24, 451-481 (1984).

Weiss, J. H., Hartley, D. M., Koh, J. and Choi, D. W., The calcium channel blocker nifedipine attenuates slow excitatory amino acid neurotoxicity. *Science*, 247 (4949 Pt 1), 1474-1477 (1990).