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Neuroprotectins A and B, Bicyclohexapeptides Protecting Chick Telencephalic Neuronal Cells from Excitotoxicity

I. Fermentation, Isolation, Physico-chemical Properties and Biological Activity

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Glutamate, an excitatory amino acid, is known to induce neurotoxicity in central nervous system under abnormal conditions such as ischemia, hypoglycemia, epilepsy, Huntington's chorea, Parkinson's disease and Alzheimer's disease. In our search for neuroprotective agents of microbial origin against excitatory neurotoxins, we have isolated two new bicyclohexapeptides, neuroprotectins A and B, together with a known compound complestatin, from the fermentation broth of *Streptomyces* sp. Q27107. Neuroprotectins protected primary cultured chick telencephalic neurons from glutamate- and kainate-induced excitotoxicities in a dose-dependant fashion.

Excitatory amino acids are known to induce considerable neurotoxicity in central nervous system under abnormal conditions. It has been demonstrated that the glutamatergic system was involved in the development of neuronal cell death followed by a variety of traumas including ischemia, hypoglycemia and epilepsy[1,2] and certain neurodegenerative diseases such as Huntington's chorea, Parkinson's disease, Alzheimer's disease and AIDS neuropathology[3~6]. The glutamatergic system is classified as two main categories depending on the agonist preference of the postsynaptic receptor system. *N*-Methyl-D-aspartate (NMDA) is one major ionotropic receptor, whereas the other comprises kainate and α-amino-3-hydroxy-5-methyl-isoxasol-4-propionic acid (AMPA) receptors. Although the mechanisms leading to nerve cell death are largely unknown, there is an overwhelming amount of literature on the importance of calcium level in cells which is elevated by the activation of NMDA receptors. It has also been known that the AMPA/kainate receptor induces the Ca^{2+} influx directly through the Ca^{2+} permeable receptors and mediates part of the excitotoxicity in the central nervous system (CNS). Kainate is a powerful neurotoxin that produces selective neuronal damage in CNS and is employed as a tool to destroy postsynaptic elements in brain with preserving presynaptic structures[7]. Kainate preferentially destroys the CA3/CA4 hippocampal formation upon intraventricular administration to rat.

NMDA antagonists had been designed for treatment of

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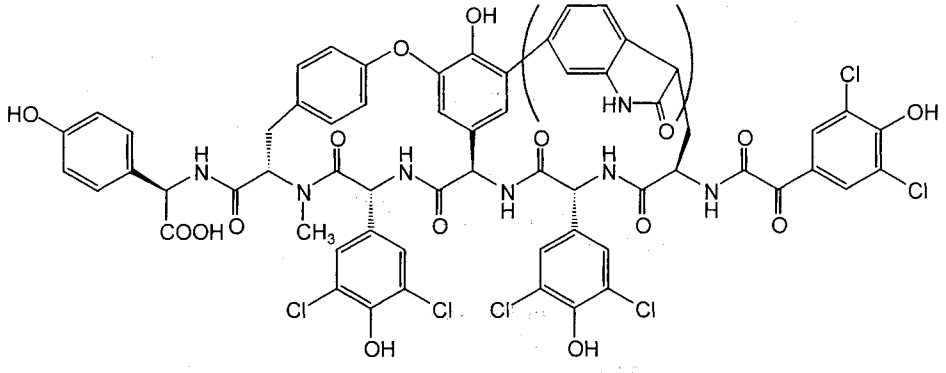
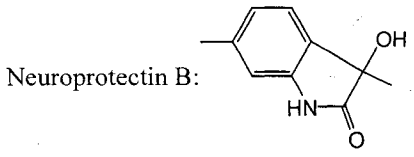
Fig. 1. Structures of neuroprotectins A and B, complestatin and chloropeptin.



Neuroprotectin A



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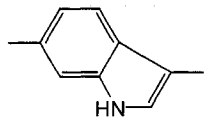
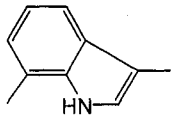


Complestatin:



Chloropeptin:

acute onset neurodegeneration such as stroke and trauma and recently tried for clinical application[8]. Unfortunately, the hopes for application of NMDA antagonists in patients so far were limited by unacceptable side effects[9~12]. The next generation of glutamate antagonists, the kainate/AMPA antagonists, turned out to be effective in reducing ischemic damage in rats subjected to global ischemia even when administered several hours after the ischemic insult[13,14]. Therefore, we have focused on screening of novel neuroprotecting compounds against kainate/AMPA-induced neurotoxicity.

During the screening of novel neuroprotective compounds against kainate-induced neurotoxicity using chick primary telencephalic cell culture, we have isolated two novel compounds, neuroprotectins A and B, along with a known compound complestatin, from the fermentation broth of *Streptomyces* sp. Q27107. Complestatin having 4-hydroxyphenylglycine and 3,5-dichloro-4-hydroxyphenylglycine moieties was previously isolated as an anti-complement and anti-HIV agent from *Streptomyces* spp.[15,16]. We herein report the fermentation, isolation, physico-chemical properties and neuroprotective activities of these compounds. The structures of neuroprotectins will be described in the accompanying paper[17].

Materials and Methods

General Experimental Procedures

Melting points were measured with a Yanaco MP-S3 micro melting point apparatus and were uncorrected. Specific optical rotations were determined with a Jasco DIP-371 digital polarimeter. UV-visible and IR spectra were recorded on Shimadzu UV-300 and Jasco A-102 spectrophotometers, respectively. Mass and high-resolution mass spectra were measured with a JEOL HX-110 spectrometer in the negative FAB mode, using glycerol matrix with polyethylene glycol as the internal standard.

Analytical Methods

TLC plates (silica gel 60 F_{254}), ODS-TLC plates (RP-18 F_{254} s) and silica gel (Kieselgel 60, 70~230 mesh) were purchased from Merck Company. ODS-TLC was developed with 50 mM citrate buffer (pH 6.0) and acetonitrile (6 : 4). HPLC was carried out using a Cosmosil C18 column (i.d. 4.6×150 mm) eluted with 45% aq. acetonitrile/0.04% TFA.

The flow rate was 0.8 ml per minute and the UV absorption of the eluate was monitored at 220 nm.

Culture of Chick Telencephalic Neuron

Dissociated telencephalic neurons were prepared from brains of 5 day-old-chick embryos according to the method of TAGUCHI[18]. The telencephalic regions of chick embryos were dissected out in Ca^{2+} and Mg^{2+}-free phosphate buffer saline (pH 7.4) supplemented with 10 mM glucose. The removed tissues were treated with 0.0125% trypsin in the same medium for 30 minutes at 37°C. The tissues were then rinsed and saturated with 2-fold diluted Eagle's minimum essential medium supplemented with 10 mM glucose, 2 mM glutamine, 2 mM $CaCl_2$, 25 mM HEPES, 60 mM NaCl, 1 mM sodium pyruvate, 50 nM Na_2SeO_3, 0.1 mM choline-Cl, 0.1 mM inositol, 5 μg/ml insulin, 20 μg/ml ovotransferin, 20 μM progesterone, 100 units/ml penicillin and 0.1 mg/ml streptomycin. The tissues were then dissociated in one ml of the same medium added with 0.2 mg DNase I. The dissociated neurons were adjusted at a density of 5×10^5 cells and plated in a volume of 200 μl per each well of 48-well microplates. These neurons were then cultured at 37°C in 5% CO_2 and in saturated humidity.

Neuroprotective Activity

After five days of culture, excitotoxicants and/or neuroprotectins were added into the culture medium. Neuroprotective activity was determined by the neuronal viability estimated by the MTT method at one day for kainate and two days for glutamate after initiation of exposure to glutamate/neuroprotectins or kainate/neuroprotectins. MTT tetrazolium salt (0.7 mg/ml) was added to neurons grown in 48-well microplates followed by incubation for 4 hours at 37°C in 5% CO_2 and in saturated humidity. The reaction medium was gently aspirated, washed with PBS buffer and then isopropanol containing 0.1 M HCl was added to solubilize the blue formazan product. Neuronal viability was estimated by quantifying soluble formazan using a Bio-Rad microplate reader at 540 nm. The activity was also confirmed by counting the number of survived neuronal cells.

Receptor Binding Assay for Glutamate Subtype Binding Sites

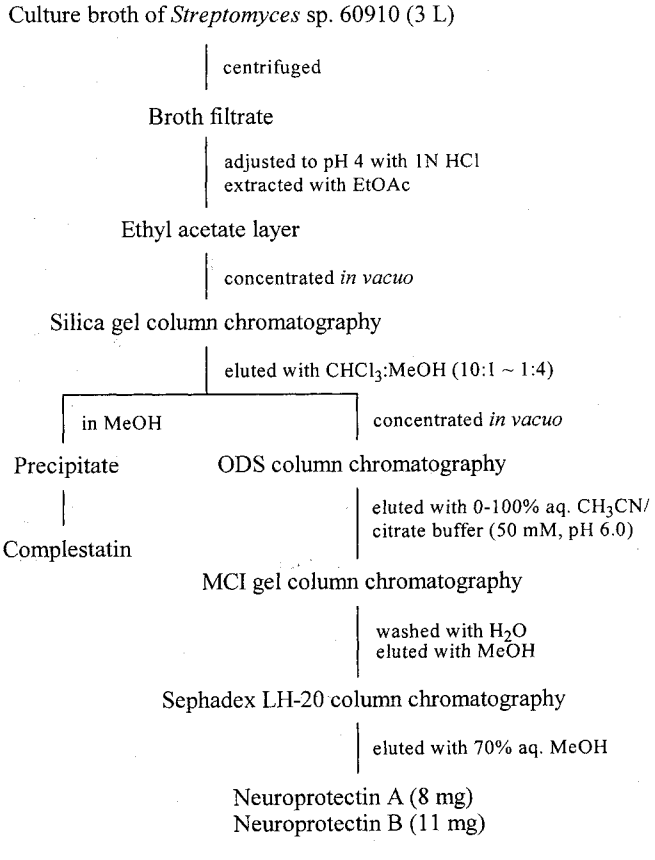
Binding experiments were performed in Panlabs (Taiwan). In brief, binding of neuroprotectin A to NMDA receptors was determined using 2 nM [^{3}H]CGP-39653 for NMDA binding site, 0.33 nM [^{3}H]MDL-105519((*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid) for strychnine-insensitive glycine receptor, 4 nM [^{3}H]TCP (tenocyclidine piperidine) for phencyclidine receptor and 2 nM [^{3}H]ifenprodil for polyamine receptor. For binding assay to AMPA or kainate receptors, [^{3}H]AMPA or [^{3}H]kainate was used. Membrane fraction from cortical tissues of male Wistar rat was incubated with specific ligands above, alone or in the presence of 10 or 30 μM neuroprotectin A.

Results and Discussion

Fermentation

A loopful of the producing strain from a mature slant culture was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of sterile seed medium consisting of soluble starch 1%, glucose 2%, soybean meal 2.5%, beef extract 0.1%, yeast extract 0.4%, NaCl 0.2%, K_2HPO_4 0.025% and $CaCO_3$ 0.2% (adjusted to pH 7.2 before sterilization) and cultured on a rotary shaker (150 rpm) at 28°C for 2 days. For the production of neuroprotectins, the seed culture was transferred in 3 ml volume into one-liter Erlenmeyer flasks containing 200 ml of the above medium

Fig. 2. Purification procedure of neuroprotectins A and B.



and cultivation was continued for 6 days under the same conditions as mentioned above.

Isolation

Neuroprotectins A and B were isolated from the culture broth of *Streptomyces* sp. Q27107. The cultured broth was centrifuged to give supernatant and mycelial cake. The supernatant was adjusted to pH 4.0 with 1 N HCl and then partitioned between EtOAc and water. The EtOAc layer was concentrated *in vacuo* and the dried residue was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH (10:1~1:4) to afford two active fractions. Addition of MeOH to one of the concentrated fractions gave complestatin. The other fraction was purified by ODS column chromatography in citrate buffer (50 mM, pH 6.0) with increasing amounts of acetonitrile. The active fraction was further purified by a column of MCI gel eluting with MeOH, followed by Sephadex LH-20 column chromatography to afford neuroprotectins A and B. The acetone extract of mycelial cake also provided complestatin. The mycelium was extracted with 70% acetone and the extract was concentrated under reduced pressure for elimination of the acetone. The resulting solution was adjusted to pH 4 and then partitioned between EtOAc and water. The EtOAc layer was chromatographed on a silica gel column and the eluate was concentrated to give complestatin by addition of MeOH.

Physico-chemical Properties

We have isolated neuroprotectins together with complestatin, which was reported as an anti-complement and anti-HIV substance. The physico-chemical properties

Table 1. Physico-chemical properties of neuroprotectins A and B.

	Neuroprotectin A	Neuroprotectin B
Appearance	Yellowish brown solid	Yellowish brown solid
MP(° C)	>300 (dec.)	>300 (dec.)
$[\alpha]_D^{19}$	+ 18.0° (*c* 0.024, MeOH)	+ 26.7° (*c* 0.012, MeOH)
Molecular formula	$C_{61}H_{45}N_7O_{16}Cl_6$	$C_{61}H_{45}N_7O_{17}Cl_6$
HRFAB-MS (*m/z*)		
found	1340.0901 $[M-H]^-$	1356.0820 $[M-H]^-$
calcd	1340.0976	1356.0925
UV λ_{max} nm (ε)		
in H_2O	216 (53000)	212 (53000)
	284 (sh.)	239 (sh.)
	359 (5000)	359 (5000)
in H_2O + NaOH	212 (72800)	210 (72800)
	265 (sh.)	255 (sh.)
	310 (6000)	312 (6400)
	346 (5300)	346 (5000)
IR ν_{max} (KBr) cm^{-1}	3360, 1660, 1480,	3380, 1660, 1630,
	1390, 1190	1480, 1190
Solubility		
soluble	DMSO, MeOH	DMSO, MeOH
insoluble	*n*-hexane, $CHCl_3$, EtOAc,	*n*-hexane, $CHCl_3$, EtOAc,
	Me_2CO, H_2O	Me_2CO, H_2O
TLC (Rf)[a]	0.17	0.20
HPLC (rt, min)[b]	10.59	7.25

[a] ODS-TLC (Merck); solvent, 40% ACN+60% citrate buffer (0.05M, pH 6.0)

[b] Column, Cosmosil C18 (4.6×150 mm); solvent, 45% aq. ACN/0.04% TFA; flow rate, 0.8 ml/min; detection, UV absorption at 220 nm.

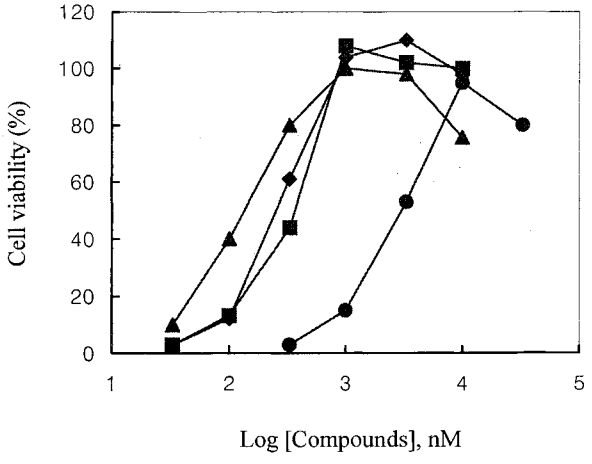
of neuroprotectins are summarized in Table 1. Neuroprotectins were obtained as a yellowish brown solid, while complestatin was purified as a yellow powder. Neuroprotectins are remarkably different from complestatin in solubility. Complestatin was easily soluble in DMSO but was insoluble in most other solvents such as MeOH, $CHCl_3$, acetone, EtOAc, BuOH and water. Neuroprotectins, however, dissolved easily in MeOH. The molecular weights of neuroprotectins A and B were determined to be 1341 and 1357, respectively, by the FAB-mass measurements in the negative mode. Their molecular formulae were established as $C_{61}H_{45}N_7O_{16}Cl_6$ and $C_{61}H_{45}N_7O_{17}Cl_6$, respectively, by high-resolution FAB-mass analyses in combination with NMR spectral data. Bathochromic shifts in the UV spectra of these two compounds by the addition of alkali suggested the presence of phenolic hydroxyl functional groups in their molecules. IR absorptions at 3360~3380 and 1660 cm^{-1} implied the presence of hydroxyl and/or amide NH and amide carbonyl functions, respectively. The molecular formulae and IR spectra suggested that these compounds belong to a peptide class of antibiotics and are structurally related to complestatin[15] and chloropeptin[16].

Biological Activities

The biological activity of neuroprotectins to protect neuronal cells from excitotoxicity was estimated by observing primary cultured chick telencephalic neurons upon treatment of excitatory neurotoxins. Exposure of 400 μM kainate in dissociated cell culture of chick telencephalic neurons resulted in neuronal cell death within 24 hours. Kainate-induced neuronal degeneration, however, was completely blocked by the addition of 0.5 μM neuroprotectins in a dose-dependent fashion as shown in Fig. 3. The ED_{50} values of neuroprotectins A, B, complestatin and a non-NMDA antagonist DNQX against kainate-induced neurotoxicity was 0.21, 0.24, 0.14 and 2.27 μM, respectively, and neuroprotectins showed about ten-times higher activity than DNQX used as control.

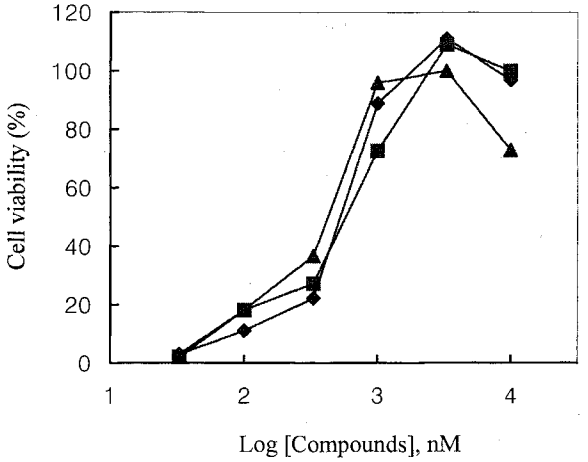
Treatment with 20 mM glutamate of neuronal culture caused neuronal cell death. The glutamate neurotoxicity was completely inhibited by the addition of neuroprotectins into culture in a dose-dependent fashion with ED_{50} values of about 0.44 μM as shown in Fig. 4. It has been known that glutamate toxicity occurs through either the activation of glutamatergic receptors (receptor-mediated pathway) and the inhibition of cystine uptake giving rise to the inability to maintain intracellular glutathione levels (oxidative pathway). In assessment by measuring inhibition effect on

Fig. 3. Protective effects of neuroprotectins, complestatin and DNQX against kainate neurotoxicity.



Chick telencephalic cell cultures were exposed continuously to 400 μM kainate alone or in the presence of indicated doses of compounds. Neuronal viability was analyzed after exposure for 24 hours to kainate by the MTT assay. Symbols indicate neuroprotectin A (◆), B (■), complestatin (▲) and DNQX (●).

Fig. 4. Protective effects of neuroprotectins and complestatin against glutamate neurotoxicity.



Chick telencephalic cell cultures were exposed continuously to 20 mM glutamate alone or in the presence of indicated doses of the compounds. Neuronal viability was analyzed after exposure for 48 hours by the MTT assay. Symbols indicate neuroprotectin A (◆), B (■) and complestatin (▲).

lipid peroxidation, neuroprotectins did not exhibit the antioxidative activity at concentrations effective to inhibit neuronal degeneration. This result implied that the neuroprotective activity of these compounds was not based on the antioxidant effect. In addition, we investigated the receptor-binding affinity of neuroprotectin A with glutamate receptor subtypes. Although neuroprotectins completely blocked neuronal degeneration induced by glutamate, they did not show any significant affinity to NMDA binding sites as did NMDA, glycine, phencyclidine and polyamine. Neuroprotectin A did not interfere with binding of non-NMDA agonists such as AMPA and kainate. To the best of our knowledge, neuroprotectins seem to inhibit neuronal cell death by excitatory neurotoxins *via* an unknown mechanism, at least not through known receptor agonist binding sites and antioxidative effects. The modes of action of these compounds are under investigation.

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