ES-242-1, A NOVEL COMPOUND FROM Verticillium sp., BINDS TO A SITE ON N-METHYL-D-ASPARTATE RECEPTOR THAT IS COUPLED TO THE CHANNEL DOMAIN

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A novel compound, ES-242-1, which binds to a site on N-methyl-D-aspartate (NMDA) receptor that is coupled to the channel domain, was isolated from the culture broth of a fungus, Verticillium sp. SPC-15898. ES-242-1 inhibited the [3H]thienyl cyclohexylpiperidine ([3H]TCP) binding to rat crude synaptic membrane fractions with an IC₅₀ value of 116 nM, but did not inhibit the [3H]kainate binding to its receptor, which is another subtype of the excitatory amino acid receptor.

The excitatory amino acids, L-glutamate and L-aspartate, are thought to play roles as excitatory neurotransmitters in the mammalian central nervous system. The receptors that mediate their actions are divided into the major three subtypes: N-methyl-D-aspartate (NMDA), quisqualate, and kainate.

The NMDA receptor is the best characterized, and is putatively involved in synaptic plasticity¹⁻³. It is possibly of physiological importance, since excessive stimulation of the NMDA receptor is thought to be involved in pathogenesis of epilepsy, stroke, anoxia, hypoglycemia, and other neurodegenerative diseases⁴. Therefore, it has been proposed that blockade of NMDA receptor-mediated neurotoxicity may be an effective approach to the treatment of brain injury. Clinical studies, however, are limited by the small number of suitable NMDA antagonists that are available. Consequently, we have sought new NMDA antagonists with potential for this therapeutic application.

During the course of our screening studies designed to obtain a new NMDA antagonist of microbial origin, we isolated a novel compound, ES-242-1, which acts on the channel domain of NMDA receptor, from the culture broth of Verticillium sp. SPC-15898. In this paper, we report fermentation, isolation and characterization of ES-242-1. Structural elucidation studies will be reported in a separate paper.

Materials and Methods

Materials
[3H]-1-[1-(2-thienyl)cyclohexyl]piperidine (TCP) and [3H]kainate were purchased from New England Nuclear. MK-801 was chemically synthesized by Dr. H. Obase and Mr. H. Kato in Pharmaceutical Research Laboratories of our company. Wistar rat brain was obtained from Nihon Seibutsu Zaibyou Center, Tokyo, Japan. L-glutamate was from Wako Pure Chemical Industries Ltd., Osaka, Japan. All other chemicals were analytical grade.

Microorganism
The producing organism, Verticillium sp. SPC-15898 (FERM BP-2604) was isolated from a soil

collected in Kanagawa Prefecture in Japan.

Culture and Medium Conditions

A loopful of spores of microorganism, grown on an agar slant, was inoculated into 10 ml of seed medium composed of V8 vegetable juice (Campbell) 20% and CaCO₃ 0.3% (pH 6.4 before sterilization) in a test tube (21 i.d. x 200 mm). The agar slant medium consisted of malt extract 2%, glucose 2%, peptone (Kyokuto) 0.1% and agar 2.0% (pH 6.5 before sterilization). The inoculated tube was incubated at 25°C. A 10%-inoculation from the above vegetative medium was added to a 300-ml Erlenmeyer flask containing 50 ml of the same medium. After cultivation for 2 days on a rotary shaker (200 rpm) at 25°C, 50 ml of the second seed culture was transferred to 2-liter Erlenmeyer flask containing 500 ml of the fermentation medium composed of glucose 2%, dried mashed potato (Yukijirushi) 2%, peptone (Kyokuto) 0.5%, K₂HPO₄ 0.05% and Mg₃(PO₄)₂·8H₂O 0.05% (pH 6.0 before sterilization) and incubated for 5 days at 25°C on a rotary shaker (200 rpm). The growth was monitored by packed cell volume (PCV) measurement. The ES-242-1 was produced mainly in mycelia, and its production was traced by measuring an inhibitory activity of [³H]TCP binding. For this measurement, 2 ml of the culture broth was sampled and centrifuged. The precipitated mycelium was extracted with 2 ml of methanol. The supernatant mixed with an equal volume of 2-propanol was treated with 4 ml of saturated ammonium sulfate solution, stirred vigorously, and centrifuged. One ml of the upper organic layer was concentrated in vacuo to dryness. The dried materials were dissolved in a same volume of methanol. In either case, 10 μl of the methanol solution was provided for the assay.

Determination of Biological Activities

TCP binding assays were performed as described⁵ with [³H]TCP and rat brain membrane as a ligand and a source of receptors, respectively. Crude synaptic membrane was prepared from rat brain according to the method of Murphy et al.⁶, but without detergent treatment. L-Glutamate was added to the solution for the binding assay to maximally stimulate the TCP receptor binding. The reaction mixture (1 ml) containing Tris-HCl (pH 7.4) 5 mM, [³H]TCP (47.8 Ci/mmol) 2.6 nM, thawed crude synaptic membrane 150~250 μg, and L-glutamate 10 μM was incubated for 30 minutes at room temperature. After which, the reaction was stopped by rapid filtration through Whatman GF/B glass filters presoaked with 0.05% polyethylene-imine. The glass filters were washed with five 3.5-ml portions of ice cold 5 mM Tris-HCl buffer (pH 7.4) by using a Brandel M-24R cell harvester. The washed filters were dried and the trapped radioactivity on the filters was determined by liquid scintillation counting in vials with 3 ml of cocktail (Omuniflour, Dupont). Non-specific binding was defined as that remaining in the presence of 50 μM of MK-801. The inhibition of [³H]TCP binding (%) was calculated as follows:

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\text{Inhibition of } [³H]\text{TCP binding} = \frac{\text{Total } [³H]\text{TCP binding in absence of assay sample} - \text{Non-specific binding}}{\text{Total } [³H]\text{TCP binding in absence of assay sample}} \times 100
\]

[³H]Kainate binding experiment was carried out in the same way as [³H]TCP binding assay. In brief, the reaction mixture (1 ml) containing [³H]kainate 5 nM, rat crude synaptic membrane 200 μg and Tris-HCl (pH 7.4) 50 mM was incubated for 1 hour on ice. L-Glutamate (1 mM) was added to the reaction mixture for the calculation of non-specific binding of [³H]kainate, after which, the reaction was stopped by filtration through the glassfilter, processed, and analyzed as described above.

HPLC Analysis of ES-242-1

The purity of ES-242-1 was determined by an HPLC system equipped with a Gilson Model 303 pump, Gilson Model 1001 UV detector and Shimadzu C-R6A Chromatopac. ES-242-1 dissolved in methanol (10 μl) was injected onto an octadecylated silica gel column (ODS-H-1151, 4.6 i.d. x 150 mm, Senshu Kagaku) and developed with 70% methanol solution at a flow rate of 1 ml/minute at room temperature, monitoring absorbance at 242 nm.
Results

Production of ES-242-1 by Fermentation

Numerous attempts to increase the yield of ES-242-1 were made; the resultant defined medium and optimum conditions for production are described under Materials and Methods. The time course of ES-242-1 production in 2-liter flask is shown in Fig. 1. The inhibitory potency against [3H]TCP binding of the culture broth initiated on day 2 and was reached maximum on day 3. The color of the broth turned red during cultivation. PCV did not change drastically. The amount of ES-242-1 produced in mycelia is approximately 10-fold higher than that in broth filtrate.

Isolation and Purification

The isolation procedure for ES-242-1 is outlined in Fig. 2. ES-242-1 was mainly purified from mycelia obtained by centrifugation (8,000 rpm, 10 minutes) of the fermentation broth. The mycelial cake was extracted with methanol. The extract was concentrated in vacuo to give aqueous solution which was then extracted with n-hexane. The hexane layer was concentrated in vacuo, to yield an oily material, which was dissolved in a small volume of chloroform and applied to a silica gel column (Wakogel C-300, 24 x 250 mm). Adsorbed material was eluted with chloroform. Fractions containing ES-242-1 were pooled and concentrated in vacuo to yield crude ES-242-1. The crude ES-242-1 thus obtained was dissolved in methanol and allowed to stand for 1 day at 4°C to yield light yellow crystalline ES-242-1 (10 mg). The mother liquor

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Fig. 1. Time course of ES-242-1 production in a 2-liter Erlenmeyer flask.
- pH, △ PCV. Inhibition of [3H]TCP binding;
- in culture supernatant or ▲ in mycelium.

Fig. 2. Purification procedure of ES-242-1.
- Culture broth (2 liters)
  - centrifuged
- Mycelial cake
  - extracted with MeOH (2 liters)
  - concd in vacuo
  - extracted with n-hexane (400 ml x 5)
  - concd in vacuo
- Oily material (348 mg)
  - silica gel column chromatography (Wakogel C-300, 100 ml)
  - eluted with CHCl₃ (400 ml)
  - concd in vacuo
- Crude ES-242-1 (37.7 mg)
  - crystallized in MeOH
- Mother liquor
  - dried up in vacuo
  - rinsed with n-hexane - acetone (9:1)
  - crystallized in MeOH
- ES-242-1 (14.8 mg)
was concentrated *in vacuo* to dryness. The material was rapidly rinsed with a mixture of *n*-hexane - acetone (9:1) to remove impurities and dissolved in methanol. The solution was kept standing at 4°C to crystallize ES-242-1 (4.8 mg). Finally, 14.8 mg of ES-242-1 was purified from 2 liters of the culture broth. Purity of ES-242-1 was determined by HPLC analysis. ES-242-1 was eluted as a single peak at a retention time of 15.3 minutes (Fig. 3), when 70% methanol solution was used as a mobile phase.

**Physico-chemical Properties of ES-242-1**

Physico-chemical properties of ES-242-1 are summarized in Table 1. ES-242-1 is readily soluble in methanol, acetone, dimethyl sulfoxide, ethyl acetate and chloroform, and virtually insoluble in water and *n*-hexane. The molecular formula of ES-242-1 was determined to be C$_{34}$H$_{36}$O$_{10}$ on the basis of HREI-MS. The UV spectrum of ES-242-1 is shown in Fig. 4. The structure of ES-242-1 was determined as shown in Fig. 5, on the basis of $^1$H and $^{13}$C NMR spectrum data. Details of the structural elucidation studies will be reported in a separate paper.
Table 2. Inhibitory activity of ES-242-1 against $[^{3}H]$TCP binding.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>IC$_{50}$ (nM)</th>
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<tbody>
<tr>
<td>$[^{3}H]$TCP</td>
<td>116</td>
</tr>
<tr>
<td>$[^{3}H]$Kainate</td>
<td>$&gt;10,000$</td>
</tr>
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Biochemical Properties

The inhibitory activity of ES-242-1 against $[^{3}H]$TCP binding is shown in Table 2 and Fig. 6. ES-242-1 displaced the $[^{3}H]$TCP binding to the synaptic membrane in a dose-dependent manner with an IC$_{50}$ value of 116 nM. The inhibitory potency of ES-242-1 was about 20-fold lower than that of MK-801 (IC$_{50}$ 5 nM) and 40-fold higher than that of ketamine (IC$_{50}$ 5.9 μM), both of which are non-competitive NMDA antagonists acting on the ion channel domain of NMDA receptor. ES-242-1 was not effective in the inhibition of the binding of $[^{3}H]$kainate, a ligand for non-NMDA type of the glutamic acid receptor, at concentrations up to 10 μM.

ES-242-1 exhibited no antimicrobial activity against Staphylococcus aureus KY 4779, Enterococcus faecalis KY 4280, Bacillus subtilis KY 4773, Escherichia coli KY 4271, Klebsiella pneumoniae KY 4275, Proteus vulgaris KY 4277, Shigella sonnei KY 4281, Salmonella typhosa KY 4278, Pseudomonas aeruginosa KY 4276 or Candida albicans KY 5011.

No inhibition of protein kinase C by ES-242-1 was observed at concentrations up to 10 μM.

Discussion

The NMDA type of excitatory amino acid receptor has been shown to possess a number of distinct sites through which its function may be regulated or pharmacologically modified. These include:

1. The agonist recognition site, at which glutamate, aspartate, and NMDA bind to open the NMDA receptor channel. The glutamate analogues 2-amino-5-phosphonovaleric acid (APV), γ-D-glutamylglycine (γ-DGG), CGS19755 (cis-4-phosphonomethyl-2-piperidine carboxylic acid), and 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonoic acid (CPP) are competitive antagonists acting on the same site;
2. An allosteric site, at which glycine regulates agonist-induced channel opening, and at which 7-chlorokynurenic acid, and HA-966 (3-amino-1-hydroxypyrrolidine-2) displace glycine binding competitively, and producing noncompetitive antagonism of receptors to NMDA; and
3. Sites with the receptor-associated ion channel for Mg$^{2+}$ and for drugs such as MK-801, ketamine, TCP, and diarylguanidine derivatives. Recent observations suggest that there may be additional site on the NMDA receptor where Zn$^{2+}$, polyamine, and tricyclic antidepressants act to modulate the properties of the NMDA receptor.

In this work we have isolated a novel compound, ES-242-1, from the culture broth of Verticillium sp., which belongs to category (3) and acts at a site of the channel domain on the NMDA receptor. Indeed, ES-242-1 displaced $[^{3}H]$TCP binding to crude synaptic membranes at a low concentration (IC$_{50}$ 116 nM) without affecting the $[^{3}H]$kainate binding. This is the first report describing the discovery of a compound acting on a site with NMDA receptor-associated ion channel from a microbial source. ES-242-1 is a new chemical entity, binds to the NMDA receptor, and as such may provide a new tool with which to understand the molecular pharmacology of this receptor. It may possess neuroprotective properties useful in the treatment of diseases involving glutamate toxicity. Further investigations concerning the biochemical and
pharmacological properties of ES-242-1 are being undertaken.

Acknowledgment

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