

# Assessment of Neuronal Cell-Based Cytotoxicity of Neurotoxins from an Estuarine Nemertean in the Han River Estuary

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A heteronemertean, *Yininemertes pratensis*, was collected in Han River Estuary, South Korea. This estuarine nemertean has been known by the local fishermen for harmful effects to the glass eels, juveniles of Japanese eel *Anguilla japonica*, migrating to fresh water. The present study confirmed the neurotoxic effects of this heteronemertean ribbon worm at the cellular level. Derivative types of neurotoxic tetrodotoxin (TTX), 5,11-dideoxy TTX ( $m/z$  288) and 11-norTTX-6(S)-01 ( $m/z$  305.97), were identified through HPLC and MALDI-TOF MS. However, significant neurotoxicity was confirmed in the fraction containing an undefined molecule corresponding to the 291.1 ( $m/z$ ) peak, when tested in rat primary astrocytes and dorsal ganglion cells. This study is the first to report neurotoxins of the estuarine nemertean, fairly abundant in the Han River estuary, and suggests the long-term monitoring of population dynamics and surveillance of the toxicity in this river estuary.

**Keywords:** Heteronemertean, *Yininemertes pratensis*, tetrodotoxin, neurotoxicity, Korean Han River estuary

## Introduction

Nemerteans, also called ribbon worms, live all over the world, mostly in marine benthic habitats, although some species have stayed to live in freshwater and land habitats. Nemerteans have no means of defense against potential predators but they rely on various toxic or noxious chemicals for their defense and predation [1–3]. They are basically carnivorous, feeding on small crustaceans, polychaetes, and mollusks [4–6], and usually range from several centimeters up to 50 cm in length.

It has been known that free-living nemerteans use their proboscis for prey capture by everting the proboscis for wrapping the prey's body tightly with explosive force [7, 8]. In this process, it seems to use neurotoxins to kill them before eating [2].

The phylum Nemertea comprises about 1,200 species [9], and about 120 species from Japanese waters [10] and 74 species from Chinese waters [11] have been reported. Only one species, *Lineus fuscoviridis* Takakura, is reported so far from Korea, inhabiting under stones on gravelly to rocky shores or sandy to muddy sediment [12]. Although many

nemerteans have been found in the various estuaries and coastal areas in South Korea, the Korean nemerteans are very poorly known.

In 2013, a large population of these nemerteans suddenly appeared from Haengjunaru in the Han River estuary, and the local fishermen have been suffering from the decline in catch of the glass eels migrating to freshwater. They complain that the neurotoxic substance secreted from the nemertean increases the mortality of juvenile eels, and has damaged the estuarine ecosystem of the Han River. Therefore, it is necessary to investigate the neurotoxins produced by the nemertean in that estuary.

Tetrodotoxin (TTX) and peptides have been found in various types of nemerteans in many previous studies [13, 14]. These toxic materials have been known to be synthesized by many *Vibrio* strains [15, 16] and thus are similar to those from blowfish, since it also has an ecosystem with *Vibrio* strains [17, 18]. TTX exists in several forms with many derivatives and the degree of toxicity depends on the derivative types. However, the nemerteans recently appeared abundantly in the Han River and several unknown neurotoxins could be included. Therefore, the

necessity of analysis of the form and influence of TTX secreted from various estuarine nemerteans inhabiting the Han River estuary has been raised.

In this study, we collected the ribbon worms from the Han River estuary and identified the TTX types by ethanol extraction, high-performance liquid chromatography (HPLC), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analyses. We also conducted cytotoxicity tests of various HPLC fractions using rat primary astrocytes and dorsal ganglion cells (DRGs).

## Materials and Methods

### Materials

Specimens of ribbon worms were carefully collected in the harvest time of *Anguilla japonica* glass eels, using the bottom net on April 30, 2013 in Haengjunaru located in the Han River estuary, South Korea (37°36'34.07"N, 126°47'56.47"E). The specimens were identified as a heteronemertean, *Yininemertes pratensis* [19], and the more detailed taxonomic study based on the specimens collected from Han River Estuary is ongoing (personal communication with Mr. TS Park, National Institute of Biological Resources, Korea). This species of heteronemertean was originally collected and described from low salinity waters (salinity 0.2~0.4‰) at Changjiang River Estuary, China [11]. The samples studied were caught at the same time with the other estuarine fishes and nereid polychaetes, as shown in Fig. 1. The hydrophobic HPLC column was purchased from YMC (Japan). Dimethyl sulfoxide and MTT solution, dispase and collagenase type A were purchased from Sigma Aldrich (USA). Dulbecco's modified Eagle's medium



**Fig. 1.** The heteronemertean ribbon worms, *Y. pratensis* [19], together with the other fishes and nereid polychaetes collected using the bottom net from Haengjunaru in the Han River estuary, South Korea (photo taken by JS Hong on April 30, 2013).

(DMEM) glucose high media, trypsin, fetal bovine serum (FBS), and Hanks' Balanced Salt Solution (HBSS) were purchased from Gibco (USA). Sprague-Dawley rats were purchased from DBL (Korea). T-75 flasks and 96-well plates were acquired from SPL Life Sciences (Korea). The ELISA reader was from Tecan (Switzerland).

### Ethanol Extraction, Fractionation, and Analysis

The specimens of the heteronemertean *Y. pratensis* [19] were collected from Haengjunaru in the Han River estuary. They were homogenized into a small size, and then put in distilled water. The samples were then filtered for 2 h through 0.25  $\mu$ m filters and autolysed, after which they were centrifuged at 8,000  $\times$ g for 30 min. Next, the supernatant was collected and dried for 24 h in an oven, after which the remaining sample was lysed in 99.9% ethanol for 2 weeks. The sample was subsequently centrifuged at 10,000  $\times$ g for 30 min to remove the pellet. Following centrifugation, the sample was freeze-dried for 24 h, and put in the second distilled water. The nemertean ethanol extracts were separated by HPLC using a hydrophobic YMC HPLC column (5  $\mu$ m 250  $\times$  20 mm). Next, 1.5 ml of nemertean ethanol extract was passed through the column at 10 ml/min and 30°C, using distilled water and acetonitrile as the mobile phase. The peak of the product was confirmed at 254 nm.

Mass analysis of the HPLC fraction was performed by MALDI-TOF. Eight HPLC fractions were analyzed, and the peak of each fraction was confirmed. The molecular peak value of the fraction was compared with those of the other reference peaks for identification.

### Cell Culture of Primary Astrocytes

Astrocytes were isolated from rat brains by primary cell culture. Sprague-Dawley rats (1 day old) were used as the experimental animals based on the previous study [20]. Briefly, brain meninges and hippocampus were removed from the cortical part of the rat brain, after which the cortical brain was pulverized using a Pasteur pipette. The samples were then filtered through a 0.7 mm cell strainer, after which cells in the filtrate were counted and seeded in T-75 flasks at  $2 \times 10^7$  per flask. After seeding, the cells were incubated under 5% CO<sub>2</sub> at 37°C for 1 week. To remove cells other than astrocytes, samples were shaken at 200 rpm for 3 days. After shaking, the flasks were centrifuged for 3 min at 1,000 rpm, and non-adhesive cells were then removed. Following removal of the pellet, the remaining supernatant was mixed with fresh medium at a 2:8 ratio in a T-75 flask. The medium exchange process was repeated and only astrocyte was finally isolated.

### Culture of Primary DRGs

DRGs were extracted from the spines of 1-day-old rats after their brains were removed. The extracted DRGs were collected in HBSS and then suspended in dissection solution (5 mg/ml dispase + 2 mg/ml collagenase type IA in HBSS). The DRGs were shaken for 10 min at 100 rpm and 37°C, after which 10 ml of HBSS

was added to the dissection solution. Next, the DRGs were pipetted using a glass pipette and then filtered through a cell strainer. Finally, the DRGs were seeded in 24-well plates at a concentration of  $1 \times 10^4$  per plate.

#### Cytotoxicity Tests of Rat Astrocytes

Cytotoxicity was confirmed by MTT assay [21]. Briefly, astrocytes in T-75 flasks were treated with trypsin after washing in PBS to remove the unattached cells. After treating with trypsin, the cells were incubated for 3 min and the supernatants were removed. The remaining pellet was placed in 3 ml glucose high-DMEM, after which astrocytes were counted using a hemocytometer. Next,  $1 \times 10^4$  cells per well were seeded in 96-well plates and the wells were filled with 200  $\mu$ l DMEM. When the concentration of the cells in the plate was sufficed, ethanol extract was then added at 0, 100, 300, 1,000, 2,000, 5,000, or 10,000 ng/ml, or HPLC fractions were added at 0.05, 0.1, or 1  $\mu$ l/ml. After 24 h, the media in the plates were removed and 100  $\mu$ l/well MTT solution was added. The samples were then allowed to react for 1 h at 37°C, after which the MTT solution was removed and crystal formazan was dissolved in 200  $\mu$ l of dimethyl sulfoxide. The optical density was confirmed at 540 nm using an ELISA reader.

#### Cytotoxicity Test of Rat DRGs

The DRG cytotoxicity was confirmed by MTT Assay. Briefly, when DRGs were sufficed in 24-well plates, the HPLC fraction was added at 0.05, 0.1, or 1  $\mu$ l/ml. After 24 h, the medium in the plate was removed and 300  $\mu$ l/well MTT was added. The samples were then allowed to react at 37°C for 1 h, after which the MTT solution was removed and crystal formazan was dissolved in 200  $\mu$ l of dimethyl sulfoxide. Finally, the optical density was measured at 540 nm using an ELISA reader.

#### Statistical Analysis

All experiments were conducted in triplicates and groups were compared by *t*-tests. *P* values of <0.05, <0.01, or <0.001 were considered to indicate significance.

## Results and Discussion

The present study was conducted to evaluate the neurotoxicity of the estuarine heteronemertean *Y. pratensis* [19], collected from Korean waters, toward nerve cells. Although the toxicity of Nemertea found has been widely reported [2, 15, 16, 22–24], the neurotoxicity of this heteronemertean isolated from the Han River estuary has not been investigated, and it has not been subjected to taxonomic analysis. Therefore, in this study, neurotoxins were extracted from the ribbon worm in the estuary of the Han River. The neurotoxins were separated into eight fractions based on their hydrophobic properties for further mass-spectrometric analysis and neurotoxicity tests.

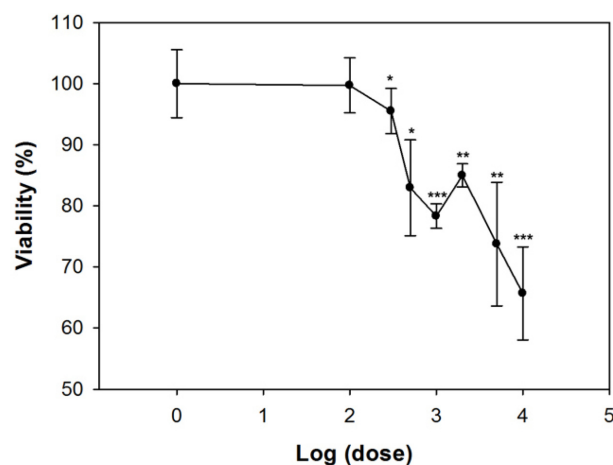
#### Cytotoxicity of the Heteronemertean Ethanol Extracts

Ethanol extracts of the heteronemertean were investigated (Fig. 2) and their HPLC fractions containing neurotoxins were tested for toxicity using astrocytes. Peptide-derived neurotoxins are known to be one of the major toxins in the ribbon worm. However, in this study, we focused on TTX toxins with strong neurotoxicity. Astrocytes are glial cells mainly distributed in the brain and spinal cord, accounting for 20% to 40% of all glial cells. These cells play significant roles in formation of the blood-brain barrier, providing metabolic nutrients to neuronal cells, and maintaining ionic balance [25]. Ethanol extracts including toxic molecules were shown to suppress cell viability of the astrocytes in a dose-dependent manner (Fig. 2).

As shown in Fig. 2, the cell viability of astrocytes decreased in response to treatment above 300 ng/ml nemertean ethanol extracts. Moreover, the cell viability dramatically decreased by more than 20% in response to treatment with 500 ng/ml, and this decrease continued in a dose-dependent fashion, eventually dropping by 47% in response to treatment with 10,000 ng/ml.

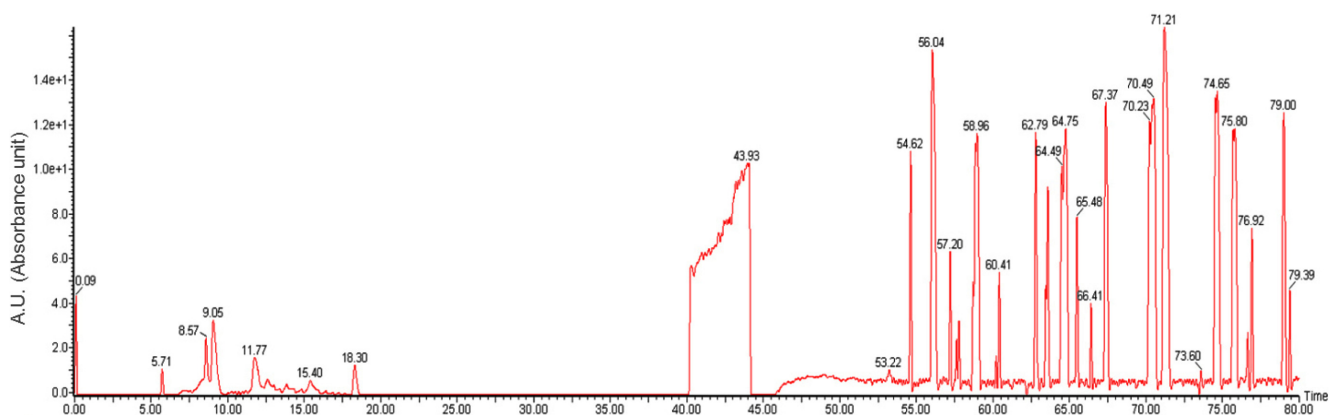
#### Fractionation of Small Molecules through HPLC

Ethanol extracts were separated using a hydrophobic YMC column to collect toxin molecules depending on their hydrophobic or hydrophilic characteristics (Fig. 3). As a result, a small portion of more hydrophilic molecules was isolated within 20 min of retention time. In previous studies, the molecular weights of TTX and its derivatives



**Fig. 2.** Viability of astrocyte exposed to ethanol extracts from the heteronemertean ribbon worm, *Yininemertes pratensis*.

The cell viability of primary astrocytes was confirmed by MTT assay. Astrocyte viability decreased in a dose-dependent manner (*p* value: \* < 0.05, \*\* < 0.01, or \*\*\* < 0.001).

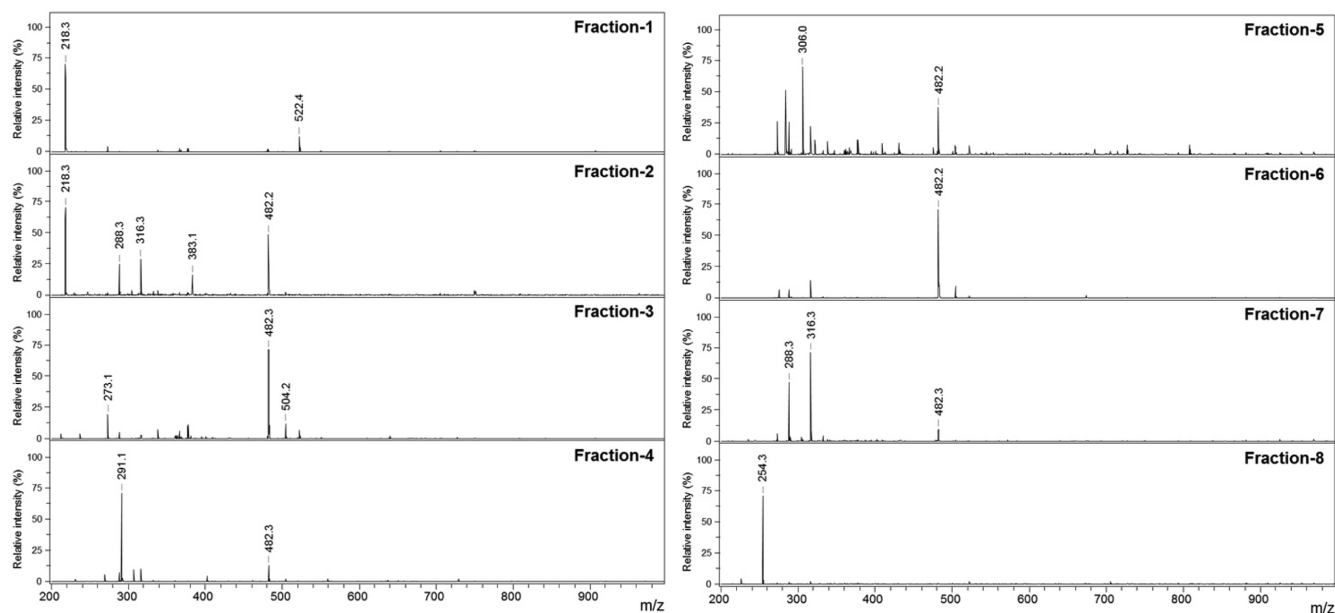


**Fig. 3.** HPLC analysis of the heteronemertean ribbon worm (*Yinemeretes pratensis*) extracts.

Acetonitrile and water were used as the mobile phases. A YMC HPLC hydrophobic column (5  $\mu\text{m}$  250  $\times$  20 mm) was used to separate the molecules, where 1.5 ml of the heteronemertean ethanol extract was passed through the column at a rate of 10 ml/min.

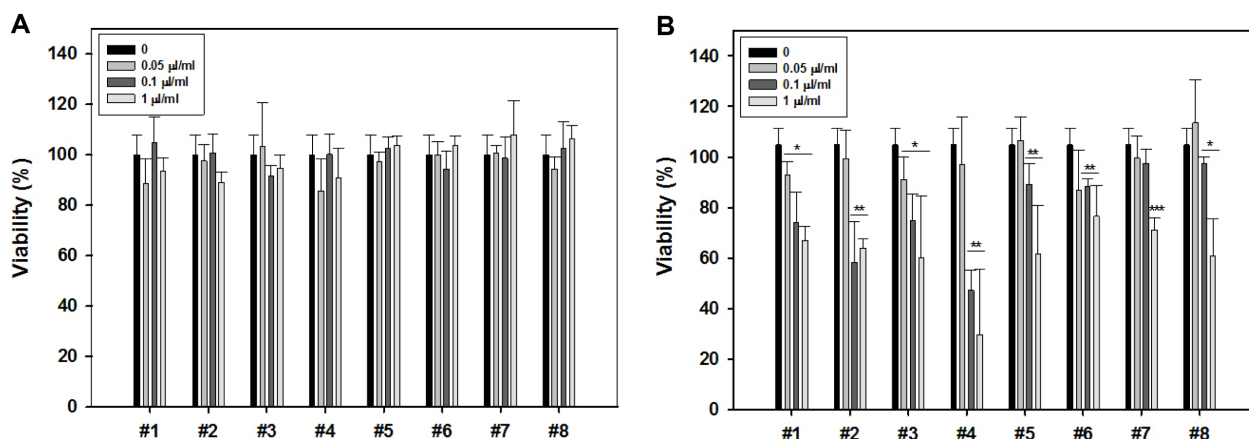
were found to range from  $m/z$  319 to  $m/z$  407, and their physicochemical properties based on molecular structures were more hydrophilic [17]. Based on the properties of TTX derivatives, HPLC fractions eluted in the first 20 min were further divided into eight fractions that were then analyzed by MALDI-TOF MS and tested for effects on astrocytes and DRGs. As shown in Fig. 3, hydrophilic materials were eluted at about 0–20 min and eight samples were obtained. MALDI-TOF-MS was then conducted to determine the molecular weight of these materials. As shown in Fig. 4, a

typical standard form of TTX with a molecular mass of 320 daltons (identical to  $m/z$  320 in the mass spectrum; Fig. 4) was not detected, but 5,11-dideoxy TTX ( $m/z$  288) and 11-norTTX-6(S)-01 ( $m/z$  305.97) were identified in fractions 2 and 5, based on peaks at  $m/z$  288 and 305.97, respectively. In a previous study, 5,11-dideoxy TTX was reported to be isolated with standard TTX from puffer fish [18]. It was also reported that TTX derivatives were toxic in an in vivo animal study, although with less toxicity than standard TTX [20].



**Fig. 4.** MALDI-TOF-MS of HPLC fractions.

The HPLC-separated fractions were analyzed by mass spectrometry to know molecular weights of molecules. Cytotoxicity to astrocytes and dorsal ganglion cells was confirmed in the eight fractions.



**Fig. 5.** Cell viability of HPLC-separated fractions.

HPLC-assisted fractions were treated in astrocytes (A) and dorsal ganglion cells (DRGs) (B). The neurotoxicity of the eight fractions in DRGs was higher than that in astrocytes ( $p$  value: \*  $< 0.05$ , \*\*  $< 0.01$ , or \*\*\*  $< 0.001$ ).

### Cytotoxicity Tests of HPLC Fractions

HPLC fractions were evaluated on astrocytes and DRGs by MTT assay. Comparison between astrocytes (Fig. 5A) and DRGs (Fig. 5B) revealed that neurotoxic effects were greater against DRGs, and that these effects occurred in a dose-dependent manner, whereas the effects against astrocytes were negligible as well as not dose-dependent. To discuss more for DRGs, the HPLC fraction showed neurotoxicity, with concentrations of 0.05  $\mu\text{l/ml}$  for fractions 1 and 3, 0.1  $\mu\text{l/ml}$  (except fraction 7), and 1  $\mu\text{l/ml}$  of all eight fractions showing decreases of cell viability. All except for the 1<sup>st</sup> and 8<sup>th</sup> fractions have a 482.3 ( $m/z$ ) peak, and the 6<sup>th</sup> fraction has the exceptionally highest intensity. In this respect, the molecule corresponding to the 482.3 ( $m/z$ ) peak is not thought to have strong neurotoxicity since the 6<sup>th</sup> fraction showed relatively high viability. Fractions 1 and 2 have 218.3 ( $m/z$ ) peaks in common. However, a molecule corresponding to this 218.3 ( $m/z$ ) peak is also not significant to DRG neurotoxicity, because the 1<sup>st</sup> fraction did not show the strongest neurotoxicity despite having the highest intensity. The 288.3 and 316.3 ( $m/z$ ) peaks are shown in the 2<sup>nd</sup> through 7<sup>th</sup> fractions. However, they are also not significant since the 5<sup>th</sup> through 7<sup>th</sup> fractions did not present higher cytotoxicity than the 2<sup>nd</sup> through 4<sup>th</sup> fractions, despite their higher intensity in 288.3 and 316.3 ( $m/z$ ) peaks. The 4<sup>th</sup> fraction had the strongest neurotoxicity to DRGs, with the cell viability decreasing by more than 70% in response to treatment with 1  $\mu\text{l/ml}$ . It has an exceptional intensity of peak at 291.1 ( $m/z$ ), and thus a corresponding molecule to this peak could be intuited to have strong neurotoxicity. Overall, we isolated hydrophilic neurotoxins from the heteronemertean ribbon worm collected in the

estuary of Han River. The results indicated that the estuarine heteronemertean, *Y. pratensis* [19], fairly abundant in Han River estuary, Korea, contained neurotoxins. However, when combined with the results of previous studies, it is suspected that their neurotoxicity varies depending on environmental circumstances and seasonal changes.

In summary, in the present study, the neurotoxicity of the heteronemertean ribbon worm *Y. pratensis* [19] collected from the Han River estuary was investigated. The samples were separated by HPLC and analyzed by MALDI-TOF-MS. The ethanol extract exhibited neurotoxicity against astrocytes. In addition, DRG viability decreased dramatically in response to treatment with the HPLC fractions with low-molecular-weight molecules. Based on these results, astrocytes are thought to be damaged by proteins or polysaccharides rather than hydrophilic small molecules. The samples were confirmed to contain the TTX derivatives 5,11-dideoxy TTX ( $m/z$  288) and 11-norTTX-6(S)-01 ( $m/z$  305.97), but neurotoxicity was highest in the sample containing an undefined molecule corresponding to 291.1 ( $m/z$ ). This is the first report of neurotoxic substances of the heteronemertean ribbon worm from Han River estuary. These findings indicate that continuous monitoring of this heteronemertean population in the estuarine region for neurotoxicity must be progressed.

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